Viral Vectors for Gene Therapy: Translational and Clinical Outlook

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Annu. Rev. Biomed. Eng. 2015. 17:63–89

The *Annual Review of Biomedical Engineering* is online at bioeng.annualreviews.org

This article's doi: 10.1146/annurev-bioeng-071813-104938

Keywords

adeno-associated virus, retrovirus, lentivirus, adenovirus, genotoxicity, vector engineering

Abstract

In a range of human trials, viral vectors have emerged as safe and effective delivery vehicles for clinical gene therapy, particularly for monogenic recessive disorders, but there has also been early work on some idiopathic diseases. These successes have been enabled by research and development efforts focusing on vectors that combine low genotoxicity and immunogenicity with highly efficient delivery, including vehicles based on adeno-associated virus and lentivirus, which are increasingly enabling clinical success. However, numerous delivery challenges must be overcome to extend this success to many diseases; these challenges include developing techniques to evade preexisting immunity, to ensure more efficient transduction of therapeutically relevant cell types, to target delivery, and to ensure genomic maintenance. Fortunately, vector-engineering efforts are demonstrating promise in the development of next-generation gene therapy vectors that can overcome these barriers. This review highlights key historical trends in clinical gene therapy, the recent clinical successes of viral-based gene therapy, and current research that may enable future clinical application.

Contents

A BRIEF HISTORY OF USING VIRAL VECTORS FOR GENE THERAPY

Pioneering Studies with Ex Vivo Gene Therapy for Immunodeficiency Diseases

The early identification of genes underlying several Mendelian disorders (1–4), followed by advances in human genetics enabled by the Human Genome Project, has provided momentum to the concept that DNA can be harnessed as a medicine to treat human disease. However, gene therapy, similar to its therapeutic predecessor monoclonal antibodies, is a field in which technological advances spanning more than a decade are now required to begin delivering on their clinical potential. In particular, strong initial successes have been enabled by the identification of well-suited vectors, advances in knowledge of human immunology, and practical approaches taken to select clinical targets.

Early pioneers in the field recognized that mammalian viruses offered the potential for efficient gene delivery, either as a biological tool or as a vehicle to treat genetic disease (5). Furthermore, in the early years of gene therapy, bringing the cells to the vector—that is, ex vivo transduction followed by cell engraftment—enabled more controlled and efficient delivery than direct in vivo administration. Thus, initial work in the field focused on blood disorders. For example, early studies used retroviral vectors based on murine leukemia virus (MLV) to transduce and then transplant T cells (6) or hematopoietic stem cells (HSCs) (7, 8) into patients to treat adenosine deaminase deficiency causing severe combined immunodeficiency (ADA-SCID). Although some responses were evident, patients continued to require ongoing enzyme replacement therapy (ERT), most likely due to insufficient engraftment of corrected stem cells.

Retroviral-mediated conditions for gene delivery were originally optimized for the transduction of cell lines whose growth rates were much different than the HSCs used in clinical applications. Furthermore, contaminants in the retroviral supernatant were discovered to affect the growth, survival, and differentiation of reimplanted HSCs. Enabled by the development of new, optimized protocols for gene delivery to HSCs, two clinical trials initiated in the late 1990s demonstrated long-term successful reconstitution of the immune system in the absence of ERT. In a 2000 report of a trial of treatment for X-linked severe combined immunodeficiency (X-SCID), 18 of 20 participants experienced restored immunity (9) without the need for ongoing ERT or a protective living environment. In the second trial, which evaluated treatments for patients with ADA-SCID and was reported in 2002, 8 of 10 treated participants had successful immune reconstitution, again without the need for ERT or a protective living environment (10, 11). These results stand in stark contrast to the standard prognosis for these patients, whose life expectancy typically does not extend beyond infancy.

In the X-SCID trial, however, 5 patients subsequently developed a clonal T cell leukemia due to integration of the retroviral vector near a proto-oncogene locus (12, 13); this could be treated with standard chemotherapy in all but one patient. Despite the occurrence (and high-profile nature) of these genotoxicity-related adverse events in the X-SCID trial, clinical outcomes in both immunodeficiency trials outperformed the standard of care, and, as such, these were the first trials to demonstrate clear evidence to support a favorable balance of risks and benefits of viral vectors. These successes have provided strong evidence to support the potential of gene therapy, and offer a foundation for future improvements in new therapeutic areas. That said, they also have highlighted two themes that have challenged gene therapy from the outset: the need for gene delivery vehicles that are both efficient and safe.

Early Setbacks for In Vivo Gene Therapy

In parallel to early clinical efforts in ex vivo gene delivery to hematopoietic cells, initial studies involving direct in vivo delivery to other tissues had more limited success. Viral vectors based on adenovirus initially offered the ostensible promise of highly efficient, therapeutic in vivo gene delivery. However, in 1999 in a trial using an adenoviral vector to treat ornithine transcarbamylase deficiency, a young patient died following systemic inflammation and multiorgan failure (14). The ensuing investigation brought to light several issues—including noncompliance with approved protocols, the underreporting of adverse events, and insufficient disclosure of conflicts of interest—and, in general, the incident led to a significant decline in enthusiasm for gene therapy, which was accompanied by reduced investments of efforts and resources during the ensuing years. Although adenoviral vectors continued to be developed for multiple indications, including anticancer therapeutics (15) and numerous vaccine efforts, much of the focus of research and development has shifted toward the development of novel vectors that could combine low genotoxicity and immunogenicity with highly efficient delivery. During this period of shifting focus, vectors based on adeno-associated virus (AAV) and lentivirus emerged as promising technological advances.

RECENT ADVANCES AND DEVELOPMENTS IN VIRAL DELIVERY VECTORS

Lentiviruses: Vector Integration with Reduced Genotoxicity

To treat most monogenic and chronic diseases, persistent expression of a therapeutic transgene is required, and this is generally possible via two mechanisms. In predominantly postmitotic cells (e.g., neurons, muscle fibers, and hepatocytes), vector DNA delivered by nonintegrating

vectors can be maintained stably in a nonintegrated (i.e., extrachromosomal) form. However, in actively dividing cells, in which nonintegrated DNA is typically diluted, vectors that integrate into the genome of the host cell, and are thus duplicated along with the host DNA during the S phase of mitosis, are typically required to enable long-term transgene expression. Unfortunately, this strategy carries the risk of insertional mutagenesis at the integration site, caused by either disrupting or inappropriately activating transcription of a nearby host gene, the latter of which occurred in the X-SCID clinical trials (12, 13).

Initial work, including the early clinical studies of treatment for ADA-SCID (6–8, 10, 11) and X-SCID (9), used vectors based on the γ-retrovirus MLV. MLV efficiently transduces dividing cells and integrates into the host genome, resulting in stable genetic correction of target cells and their progeny (as reviewed in 16). In particular, MLV integrates in a pseudorandom fashion within cellular genomes, with a preference for actively transcribed DNA (17, 18), especially near the transcriptional start site of genes and within hot spots that are enriched for proto-oncogenes and growth-controlling genes (19). Furthermore, MLV integration favors regulatory elements, regions near the transcription start site of genes, CpG islands, conserved noncoding sequences, and genes that are actively transcribed within the targeted cell type (20). MLV integration sites have been shown to correspond to physical properties of the DNA itself, including the outward-facing major grooves of nucleosome-wrapped DNA (21). In the 5 of 20 participants across the two X-SCID trials who developed clonal T cell leukemia, integration-site analysis of the transformed cells showed that 3 cases carried a single insertion near the *LMO2* gene locus, a fourth featured two integration sites near the proto-oncogenes *LMO2* and *BMI1*, and the fifth carried an insertion at another proto-oncogene, *CCND2* (12, 13, 22, 23). The adverse outcomes are believed to be due to a combination of host proto-oncogene activation as well as the subsequently discovered oncogenic nature of the transgene, which encodes the common γ_c chain cytokine-receptor subunit (24). Due to the former issue, much attention has since been focused on addressing insertional mutagenesis, or the genotoxicity, posed by integrating vectors.

One strategy for reducing the genotoxicity of integrating vectors is to develop self-inactivating, or SIN, vectors, in which the enhancer or promoter of the long terminal repeat (LTR) is deleted, theoretically decreasing the risk of activating nearby genes. However, SIN-MLV vectors suffer decreased transduction compared with their full LTR counterparts in HSCs (25). That said, another recent report has suggested that a SIN-MLV vector exhibited a reduced preference for growth-related genes and transcriptional start sites in human keratinocytes compared with corresponding full LTR vectors (26). These and related strategies, such as the use of chromatin insulators or alternative promoters, may lead to novel MLV vectors that have improved genotoxicity profiles and, therefore, increased clinical utility (12).

In parallel, relatives of γ-retroviruses have been developed into highly promising gene delivery vehicles. In 1996, the human immunodeficiency virus (HIV) was first engineered into an integrating lentiviral gene delivery vector capable of efficient delivery to both mitotic and nondividing cells (27), and other nonhuman lentiviruses have since been converted into vectors. Both γ-retroviruses and lentiviruses are single-stranded RNA viruses that package two copies of positive-strand RNA containing three genes: *gag* (which encodes structural proteins), *pol* (which encodes the reverse transcriptase, integrase, and protease enzymes that are packaged with the RNA strands inside the virus), and *env* (which encodes the envelope proteins that coat the virus) (28) (**Figure 1*a***). In addition to genes found in γ-retroviruses, HIV and other lentiviruses carry six genes encoding accessory proteins, named *tat*, *rev*, *vpr*, *vpu*, *nef*, and *vif* (28) (**Figure 1*b***). Initially, gene delivery vectors based on HIV were packaged using plasmids encoding *gag*, *pol*, and the accessory genes; a separate *env* plasmid; and the gene of interest contained inside the HIV LTR (28). Subsequent efforts led to the development of third-generation lentiviral vector systems that were stripped of

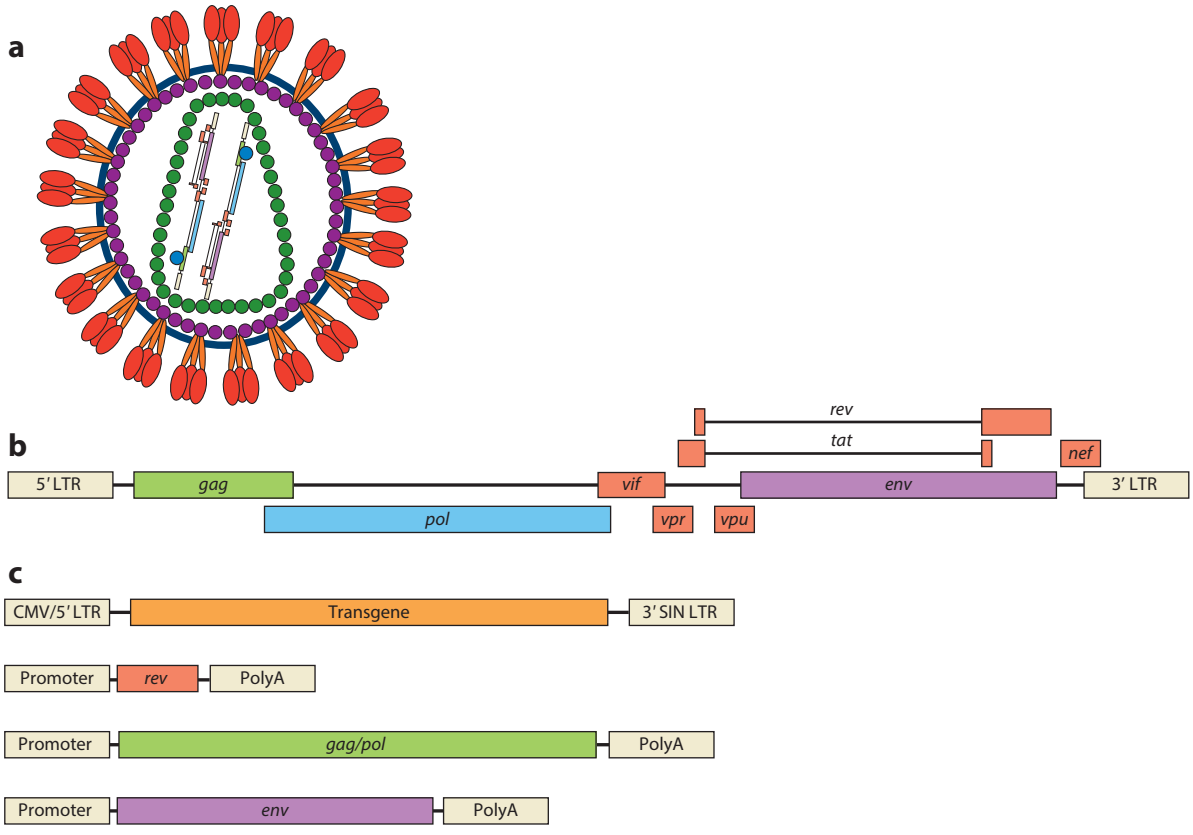

Figure 1

Lentivirus and lentivirus vector. (*a*) Schematic of lentivirus. Two copies of positive-strand RNA are surrounded by the protein capsid and envelope. (*b*) The RNA genome of lentivirus. The genome encoding the *gag*, *pol*, *env*, and accessory protein genes is flanked by LTRs. (*c*) Genetic components of lentivirus vectors. The therapeutic transgene is inserted between the viral LTRs, which also function as a promoter sequence. Lentivirus *gag/pol*, *env*, and *rev* RNA sequences are supplied in *trans* to produce the vector. Abbreviations: CMV, cytomegalovirus; LTR, long terminal repeat; PolyA, polyadenylation sequence; SIN, self-inactivating.

many accessory viral proteins and *cis* DNA elements, leading to the development of SIN vectors with improved safety (29) (**Figure 1*c***).

Furthermore, although some similarities exist between the integration profiles of γ-retroviruses and lentiviruses, there are also important differences. Analogous to MLV, a large number of studies have shown that HIV integration occurs in actively transcribed genes, with some regions of hot-spot activity (30–33). Also similar to MLV, lentiviral integration is associated with physical properties of genomic DNA, such as the outward-facing major grooves of a nucleosome (21, 34, 35). HIV integration has also been associated with histone modifications, such as H3 acetylation, H4 acetylation, and H3K4 methylation; however, it appears to be disfavored at sites of H3K27 trimethylation and DNA CpG methylation (34). As a result of the latter property, lentiviral vectors (unlike γ-retroviruses) do not exhibit a preference for the 5′ regions of genes, and integration is actually disfavored at, or upstream of, the transcription start site (20, 21, 31). In addition, unlike MLV, HIV does not favor integration into DNase I–sensitive sites (36). The virus instead prefers to integrate in downstream regions within transcribed genes, which are significantly safer regions compared with promoter elements. Differences between γ-retroviral and lentiviral vector

integration can apparently be largely attributed to the Integrase and, to a lesser extent, Gag proteins, as transferring these MLV proteins into an HIV background resulted in hybrid vectors exhibiting integration patterns similar to MLV (36). The proposed model for HIV integration involves viral Integrase recruiting the transcriptional-mediator protein LEDGF/p75 (37–40), which through its N-terminal domain directs the integration machinery to active transcription units (41), and thereby appears to, at least partially, mediate the preference for integrating in active genes (42).

Studies comparing the genotoxicity of γ-retroviral and lentiviral vectors in mouse models have shown that, whereas γ-retroviral vectors can insert into growth-control genes and thereby trigger dose-dependent acceleration of tumor formation, using lentiviral vectors did not lead to analogous insertion in such growth-control genes, or to clonal expansion or tumor growth (43). Another study in *Cdkn2a*$^{-/-}$ tumor-prone mice demonstrated that substantially greater lentiviral-integration loads are required to approach the same oncogenic risk as γ-retroviral vectors, and identified the γ-retroviral LTRs as a major determinant of genotoxicity (44). That said, recent studies have indicated that insertional mutagenesis has a role in the natural progression of HIV infection (45).

In general, the integration properties of lentiviral vectors may be translating toward improved clinical safety. Although one participant in a clinical trial who was treated with a lentiviral vector for β-thalassemia (a trial discussed in greater detail in the Recent Clinical Successes and Ongoing Promising Studies: Retroviral and Lentiviral Vectors section) developed a clonal expansion in which the vector had integrated within the host *HMGA2* gene, which has been associated with HSC expansion, the participant has remained healthy for more than 4 years. In addition, studies in mice have shown that integration sites near growth-control genes, such as *HMGA2*, were not favored initially or through selective pressure (46, 47). Thus, lentiviral vectors are promising and apparently safe vehicles for clinical gene therapy.

Adeno-Associated Viruses: Enhanced In Vivo Gene Delivery

Although lentiviral vectors offer strong potential for ex vivo gene delivery, their biodistribution properties and neutralization by serum components such as complement render their use as a systemic gene delivery vehicle challenging (48). In parallel, work with vectors based on AAV has revealed their strong potential for in vivo gene delivery. AAV is a nonpathogenic parvovirus composed of a 4.7 kb single-stranded DNA genome within a nonenveloped, icosahedral capsid (49) (**Figure 2*a***). The genome contains three open reading frames (ORF) flanked by inverted terminal repeats (ITRs) that function as the viral origin of replication and the packaging signal (**Figure 2*b***). The *rep* ORF encodes four nonstructural proteins that have roles in viral replication, transcriptional regulation, genomic integration, and virion assembly. The *cap* ORF encodes three structural proteins (VP1–3) that assemble to form a 60-mer viral capsid (49). Finally, an ORF that is present as an alternate reading frame within the *cap* gene produces the assembly-activating protein, a viral protein that localizes AAV capsid proteins to the nucleolus and functions in the capsid assembly process (50, 51). In recombinant versions of AAV, a gene of interest is inserted between the ITRs in place of *rep* and *cap*, and the latter are provided in *trans*, along with helper viral genes, during vector production (52) (**Figure 2*c***). The resulting vector can transduce both dividing and nondividing cells, with stable transgene expression for years in the absence of helper virus in postmitotic tissue. There are 11 naturally occurring serotypes and more than 100 variants of AAV, each of which differs in its amino acid sequence, particularly within the hypervariable regions of the capsid proteins, and, thus, also differ somewhat in their gene delivery properties (53, 54). Recombinant vectors were initially clinically tested in trials for indications including hemophilia B (55), rheumatoid arthritis (56), and cystic fibrosis (57). Clinical development is discussed in more detail in the Recent Clinical Successes and Ongoing Promising Studies: Adeno-Associated Virus Vectors section.

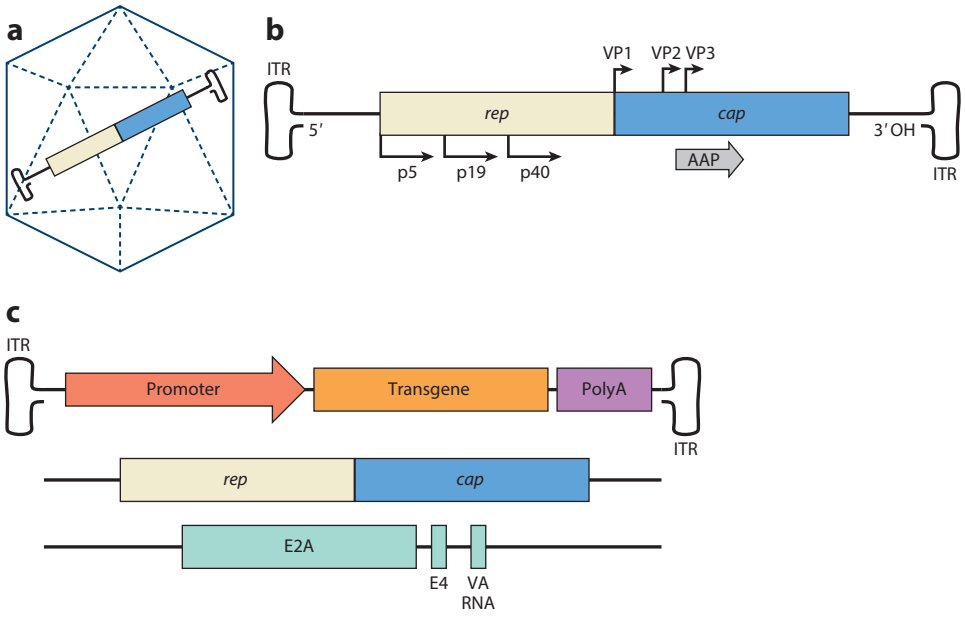

Figure 2

AAV and its vector. (*a*) Schematic of AAV. A single-stranded genome is surrounded by the protein capsid. (*b*) The DNA genome of AAV. The 4.7 kb genome encoding the *rep*, *cap*, and *aap* open reading frames is flanked by ITRs. (*c*) Genetic components of AAV vectors. The therapeutic transgene, along with associated promoter and polyadenylation sequences, is inserted between the viral ITRs. AAV *rep* and *cap*, and adenovirus E2A, E4, and VA RNA sequences are supplied in *trans* to produce the vector. Abbreviations: AAP, assembly-activating protein; AAV, adeno-associated virus; ITR, inverted terminal repeat; PolyA, polyadenylation sequence; VA, viral-associated.

Although wild-type AAV serotype-2 (AAV2) is capable of *rep*-dependent integration in a specific locus called *AAVS1* on chromosome 19 (58), *rep* is deleted from recombinant AAV vectors that are used for gene therapy, which, therefore, are not capable of site-selective genomic integration. Instead, after entry into the cell and second-strand synthesis, AAV forms high molecular weight concatemers or circular DNA (59, 60); these persist extrachromosomally in nondividing cells. The genomic integration of recombinant AAV vectors occurs at a low background frequency, with studies in cell culture and in muscle cells estimating a 0.1–0.5% frequency, and other experiments suggesting potentially higher frequencies of 5–10% in some tissues, such as mouse liver (61). However, a recent study examining recombinant AAV integration in participants in clinical trials found that the use of alipogene tiparvovec (Glybera, uniQure, Amsterdam, the Netherlands) (discussed in greater detail in the Recent Clinical Successes and Ongoing Promising Studies: Adeno-Associated Virus Vectors section) results in low-level integration into random sites and is not associated with toxicity (62).

Additional Gene Delivery Vehicles

In addition to lentivirus vectors and AAV vectors, gene delivery technologies involving adenovirus, herpes simplex virus, and poxvirus (most commonly vaccinia virus) vectors have been utilized in

an increasing number of clinical studies. These vectors are generally nonintegrating and are not associated with a known risk of insertional mutagenesis or genotoxicity.

Adenovirus. Adenoviruses are a class of DNA viruses with a double-stranded 34–43 kb genome, which employs alternative splicing to encode genes in both the sense and antisense orientations (63). The adenovirus genome is flanked by two ITRs and contains eight transcription units: early units (E1, E2, E3, E4, and E5), units with delayed expression after viral replication is initiated (IX and IVa2), and a late unit (subdivided into L1, L2, L3, L4, and L5 genes) (63). Similar to recombinant AAV vectors, the adenovirus genome remains in an extrachromosomal form following infection. There are many different serotypes of adenovirus, including 51 found in humans (63); Ad5 is the most prevalent, and 45–80% of the population harbors neutralizing antibodies against this serotype due to natural infections, which typically date back to infancy (63). Standard recombinant adenoviral vectors have the E1 unit deleted, and removal of E3 can create vectors capable of packaging up to 7.5 kb of foreign DNA (63). Vectors have also been created with both E1 and E4 units deleted, which results in lower immune responses (63). Finally, in helper-dependent, or gutless, adenoviral vectors, all adenoviral genes are deleted, and these are provided in *trans* by a helper adenovirus for production. Although these have a large carrying capacity of more than 30 kb, they are currently challenging to manufacture (64). Another concern with adenoviral vectors in general is that the particles themselves elicit cellular inflammatory responses, compared with, for example, relatively inert AAV virions (65).

Herpes simplex virus. Herpes simplex virus (HSV) is an enveloped virus with a double-stranded DNA genome more than 150 kb in length, which is divided into long and short unique segments (U_L and U_S) and flanked by inverted repeated sequences (TR_L/IR_L and TR_S/IR_S) (66). Approximately 90 genes are encoded by the HSV genome, approximately half of which are nonessential and can be eliminated in recombinant vectors (66). There are currently three types of HSV vectors used for gene delivery and gene therapy applications. First, amplicons are gene delivery systems packaged through transfection into producer cells of plasmids containing the HSV origin of DNA replication, HSV cleavage-packaging sequences, and transgene(s) of interest. The plasmids form head-to-tail linked concatemers that are packaged into the viral particles, which can, in general, accommodate large fragments of foreign DNA (66). In a second system, deletion of genes necessary for the lytic cycle of HSV can create replication-defective vectors that are less toxic and elicit a smaller immune response than wild-type HSV vectors (66). Several versions of replication-defective HSV vectors have been created by deleting various combinations of the immediate–early genes that otherwise lead to expression of the HSV infected cell proteins: ICP0, ICP4, ICP22, ICP27, and ICP47 (66). Finally, replication-competent HSV vectors are attenuated versions of HSV that contain the genes necessary for replication in vitro, but lack the genes necessary for replication in vivo (66). Replication-competent vectors have been used as oncolytic therapies and as vaccines, where the set of deleted genes must lead to the appropriate balance of replicative attenuation for safety and lytic activity for tumor toxicity or vaccine immunogenicity, respectively.

Vaccinia virus. Vaccinia is a member of the poxvirus family with an approximately 200 kb linear double-stranded DNA genome (67). Vaccinia was utilized in the vaccine that led to the eradication of smallpox (67). Recombinant vaccinia vectors are capable of packaging up to 25 kb of foreign DNA, and deletion of the thymidine kinase gene inhibits replication of the virus in nondividing cells (68). Vaccinia's ability to selectively replicate in dividing cells has been harnessed for the development of oncolytic viral vectors. For example, the oncolytic vaccinia variant JX-594 has been engineered for transduction of, and replication specifically in, cancer cells in which the

epidermal growth factor receptor–Ras pathway is activated. In addition to the cancer-specific replication, JX-594 expresses the granulocyte–monocyte colony–stimulating factor transgene for additional stimulation of antitumoral immunity (69–71). The results of clinical trials utilizing these vectors are described in the Recent Clinical Successes and Ongoing Promising Studies: Other Gene Therapy Approaches section.

RECENT CLINICAL SUCCESSES AND ONGOING PROMISING STUDIES

Retroviral and Lentiviral Vectors

As described previously, retroviral vectors have been used with some success in clinical trials involving ex vivo hematopoietic gene delivery (**Table 1**). In greater detail, clinical trials for X-SCID or ADA-SCID have involved in vitro transduction of $CD34^+$ HSCs with MLV retroviral vectors encoding the γ_c gene or the ADA gene, respectively (9–11). In two patients who had received γ_c-expressing HSCs for the treatment of X-SCID, T cells and natural killer cells expressing the γ_c transgene were detected 10 months posttreatment, and T, B, and natural killer cells reached counts and functions comparable to those in controls with a normal immune system (9). In addition, two patients administered with ADA-expressing HSCs in the first ADA-SCID trial, published in 2002, showed sustained engraftment of HSCs that underwent differentiation into multiple lineages, increased lymphocyte counts, improved immune functions, and reduced toxic metabolites (10). Of the 10 patients who participated in the second ADA-SCID trial, published in 2009, 8 no longer required ERT for ADA, 9 had increased T cell counts and function, and 5 elicited antigen-specific antibody responses to vaccines (11).

Building upon this promising foundation, lentiviral vectors have since emerged as a strong vehicle for the transduction of hematopoietic cells. Even before the discovery that brain microglia are of hematopoietic origin, research had begun to show that the cerebral deficits resulting from lysosomal storage disorders could be ameliorated through the transplantation of HSCs expressing normal copies of the mutated lysosomal enzyme. One such lysosomal storage disorder—X-linked adrenoleukodystrophy (ALD), caused by mutations to the fatty acid transporter encoding the *ABCD1* gene—is characterized by the tissue accumulation of long chain fatty acids that damage myelin, resulting in eventually fatal neurodegeneration. In a trial of treatment for ALD, two patients were administered HSCs that had been transduced with a SIN G glycoprotein from the vesicular stomatitis virus (VSV-G) pseudotyped lentivirus encoding the *ABCD1* gene (72). Demyelination in the brain was halted by 14 months posttherapy in 1 patient and 20 months posttherapy in the second patient; by 36 months posttherapy no new lesions had been observed in either patient (72).

In another trial, HSCs transduced ex vivo with a SIN lentiviral vector encoding β-globin were administered to a patient with severe β^E/β^0-thalassemia (46). Almost 3 years after this gene therapy, the patient maintained blood hemoglobin levels in the range of 9–10 g/dl (only slightly below the average adult hemoglobin level) and remained independent of blood transfusions (46). Furthermore, three patients in a Phase I/II clinical trial of treatment for Wiskott–Aldrich syndrome (WAS) were administered HSCs transduced with a lentiviral vector encoding the WAS gene. The engineered HSCs exhibited robust, stable, and long-term engraftment in the patients, and patients showed improved T cell function and protection from severe infections (73). Importantly, there was no evidence of clonal expansion during 18 months posttherapy (73). Using a similarly engineered HSC approach, a Phase I/II clinical trial of treatment for metachromatic leukodystrophy (MLD) administered HSCs transduced with a lentiviral vector encoding the arylsulfatase A

Table 1 Summary of clinical trials using retroviruses by disease

Vector type	Transgene	Clinical phase	Route of administration	Clinical trial identifier	Reference(s)
Adenosine deaminase deficiency–severe combined immunodeficiency (ADA-SCID)					
MLV retrovirus	ADA	NA	Ex vivo to T cells	NA	6
MLV retrovirus	ADA	NA	Ex vivo to HSCs	NA	7, 8
MLV retrovirus	ADA	Phase I/II	Ex vivo to HSCs	NCT00598481 NCT00599781	10, 11
X-linked severe combined immunodeficiency (X-SCID)					
MLV retrovirus	γc	Phase I	Ex vivo to HSCs	NA	9
β-Thalassemia					
SIN lentivirus	β-globin	Phase I/II	Ex vivo to HSCs	NA	46
X-linked adrenoleukodystrophy (ALD)					
SIN, VSV-G lentivirus	ABCD1	Phase I	Ex vivo to HSCs	NA	72
Wiskott–Aldrich syndrome (WAS)					
SIN, HIV-derived lentivirus	WAS	Phase I/II	Ex vivo to HSCs	NCT01515462	73
Metachromatic leukodystrophy (MLD)					
SIN, VSV-G lentivirus	Arylsulfatase A gene	Phase I/II	Ex vivo to HSCs	NCT01560182	74
Chronic lymphocytic leukemia (CLL)					
SIN lentivirus	Chimeric antigen receptors	Phase I	Ex vivo to T cells	NCT01029366 NCT00466531	75–77
B-cell acute lymphoblastic leukemia (ALL)					
SIN lentivirus	Chimeric antigen receptors	Phase I	Ex vivo to T cells	NCT01044069	77, 78

Abbreviations: γc, gamma-chain protein of the interleukin receptor; HSCs, hematopoietic stem cells; MLV, murine leukemia virus; NA, not applicable; ABCD1, adenosine triphosphate (ATP)-binding cassette, subfamily D (X-linked adrenoleukodystrophy), member 1; SIN, self-inactivating; VSV-G, G glycoprotein of the vesicular stomatitis virus.

gene (74). All three patients demonstrated high-level stable HSC engraftment in the bone marrow and peripheral blood, and they maintained normal motor and cognitive development for at least 2 years posttherapy (74).

Lentiviral vectors have also been clinically utilized to treat leukemia. Chemotherapy-refractory chronic lymphocytic leukemia (CLL) and relapsed B-cell acute lymphoblastic leukemia (ALL) are generally resistant to current cancer therapies. As an alternative, several clinical trials have explored the use of T cells engineered ex vivo to express chimeric antigen receptors (CAR) composed of a costimulatory domain (which promotes T cell proliferation and survival) and a T cell receptor ζ chain that targets CD19 (75–78). The T cells expressing this CAR19 transgene mediated a CD19-specific immune response against leukemia cells expressing this antigen. In the first trial, two of three patients achieved complete remission of CLL (75, 76). Additionally, the third patient achieved partial remission, in which the CLL cells remained in the bone marrow but were significantly reduced in the peripheral blood (76). In another trial, four of five patients achieved complete remission of ALL (78). In a third clinical study, nine patients showed mixed effects from the CD19 T cells, with disease stabilization or reduction occurring only in patients who had received chemotherapy prior to the T cell infusion (77). These landmark studies have helped extend the reach of gene therapy to cancer, promising a new generation of cancer immunotherapies.

Future research will elucidate whether T cell therapy may be more effective in patients with a low tumor burden or prior chemotherapy (77), explore whether this approach can be effective for solid tumors, and investigate whether tumor-specific antigens can be identified for other cancer targets.

Adeno-Associated Virus Vectors

In parallel, AAV has yielded successful results in an increasing number of clinical trials (**Table 2**). On November 2, 2012, the European Medicines Agency approved alipogene tiparvovec (Glybera), albeit under exceptional circumstances that require an additional postmarketing study to further demonstrate efficacy. Glybera is an AAV1-based vector that delivers human lipoprotein lipase (LPL) complementary DNA (cDNA) to muscle cells via intramuscular injection. This is the first gene therapy treatment to be approved in Western nations. In clinical trials, AAV1 vectors encoding LPL^{S447X} (a gain-of-function variant) were administered via intramuscular injection at 30–70 sites in the upper and lower limbs, and subsequent gene expression resulted in transient decreases in mean triglyceride levels and a decreased incidence of pancreatitis in virtually all patients. Furthermore, long-term evaluation of these patients has shown a strong safety profile, persistent gene expression and protein activity, and continued therapeutic benefits (79–81).

In addition, the retina has been a very promising target for AAV gene therapy as it is surgically accessible, is relatively immune privileged, and can be transduced with relatively small doses of the vector. In three landmark Phase I clinical trials for treatment of the human blinding disease Leber's congenital amaurosis type 2 (LCA2), more than 30 patients who received a subretinal injection of AAV2 encoding RPE65—the enzyme that isomerizes the retinal chromophore to the form needed for photoreceptors to sense light—have shown substantial and sustained improvements in both subjective and objective measures of vision (82–84). In addition, functional improvements were sustained throughout a subsequent 3-year follow-up period (85). Furthermore, 3 of 12 patients have received vector readministration in their other eye, which has resulted in improved retinal and visual function, and did not elicit an immune response against the vector (86). A Phase III clinical trial is currently under way for treatment of LCA2. In a recent Phase I/II clinical trial for choroideremia, 6 patients who received subretinal injection of AAV2 encoding the Rab1 escort protein, which is mutated in this disorder, demonstrated increased retinal sensitivity and a gain in visual acuity (87).

Furthermore, in Phase I clinical trials for hemophilia B, AAV2 and AAV8 vectors encoding the transgene for human Factor IX (FIX) were delivered via intramuscular (AAV2), intrahepatic (AAV2), or intravenous (AAV8) injection (55, 88, 89). All three trials demonstrated safety and progressively improved efficacy. The results of the first trial demonstrated that intramuscular delivery was incapable of providing sufficient levels of FIX expression. The second trial, exploring the delivery of AAV2 to the liver, showed that AAV gene therapy was capable of eliciting transient expression of therapeutic levels of FIX; however, apparent $CD8^+$ T lymphocyte responses to major histocompatibility complex I cross-presentation of AAV capsid antigens on transduced hepatocytes subsequently attenuated FIX expression. However, in a later Phase I/II clinical trial, intravenous delivery of AAV8-FIX, coupled with short-course glucocorticoid therapy as necessary to suppress the $CD8^+$ lymphocyte response, enabled FIX protein to be secreted into blood at levels sufficient to alleviate the patients' bleeding phenotype (89).

AAV vectors have also demonstrated safety in clinical trials for Canavan's disease, muscular dystrophy, and α-1-antitrypsin deficiency (90–95). That said, these clinical trials did not achieve efficacy, highlighting the need for continued improvements in gene delivery vectors, transgenes, routes of administration, and immune interactions.

Table 2 Summary of clinical trials using adeno-associated virus (AAV) by disease

AAV serotype	Transgene	Clinical phase	Route of administration	Clinical trial identifier	Reference(s)
Hemophilia B					
AAV2	Factor IX	Phase I/II	Intramuscular	NCT00076557	55, 88, 89
		Phase I	Hepatic	NCT00515710	
AAV8		Phase I/II	Intravenous	NCT00979238	
Rheumatoid arthritis					
AAV2	TNF receptor–antibody fusion	Phase I/II	Intraarticular	NCT00617032 NCT00126724	56
Cystic fibrosis					
AAV2	CFTR	Phase I/II	Aerosol	NCT00004533	57
Lipoprotein lipase deficiency					
AAV1	LPL	Phase I/II/III	Intramuscular	NCT01109498	79–81
				NCT00891306	
Leber's congenital amaurosis					
AAV2	RPE65	Phase I/II	Subretinal	NCT00516477	82–86
				NCT00643747	
				NCT00481546	
Choroideremia					
AAV2	REP1	Phase I/II	Subretinal	NCT01461213	87
Canavan's disease					
AAV2	Aspartoacylase	Phase I	Intracranial	NA	91
Muscular dystrophy					
AAV1/AAV2 chimera	Microdystrophin	Phase I	Intramuscular	NCT00428935	90
AAV1	α-Sarcoglycan	Phase I	Intramuscular	NCT00494195	94, 95
α-1-Antitrypsin deficiency					
AAV2	α-1-Antitrypsin	Phase I/II	Intramuscular	NCT00377416	92, 93
AAV1				NCT00430768	
Severe heart failure					
AAV1	SERCA2a	Phase I/II	Coronary artery infusion	NCT00454818	96, 97
Parkinson's disease					
AAV2	GAD	Phase I/II	Intracranial	NCT00195143 NCT00454818	99, 100
	Neurturin	Phase I/II		NCT00252850	101
				NCT00400634	
	AADC	Phase I		NCT00229736	102, 103
Wet age-related macular degeneration					
AAV2	anti-VEGF	Phase I/II	Subretinal	NCT01494805	
		Phase I	Intravitreal	NCT01024998	

Abbreviations: AADC, L-amino acid decarboxylase; CFTR, cystic fibrosis transmembrane conductance regulator; GAD, glutamic acid decarboxylase; LPL, lipoprotein lipase; NA, not applicable; REP1, Rab1 escort protein; RPE65, retinal pigment epithelium-specific protein 65 kDa; SERCA2a, sarcoplasmic reticulum calcium ATPase 2a; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

In addition to treating monogenic diseases, AAV gene therapy has shown promising signs for treatment of several idiopathic or complex diseases. The Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) clinical trials applied AAV gene delivery to treat class III and IV heart failure. In the Phase I and II trials, AAV1 vectors encoding SERCA2a [sarcoplasmic reticulum calcium adenosine triphosphatase (ATPase)] were delivered via intracoronary injection to patients with advanced heart failure (96, 97). Improvement was seen in almost all patients, as measured by a decrease in symptoms, improvement in functional status, the presence of biomarkers, and left ventricular function (96, 97). In the Phase II trial, there were also significant increases in the time to clinical events, a decreased frequency of cardiovascular events, and a decrease in the mean duration of cardiovascular hospitalizations during the 12 months following gene therapy administration (97). Preclinical studies using the S100A1 transgene, a different regulator of calcium signaling, have also been promising (98).

Several clinical trials utilizing AAV vectors to treat Parkinson's disease (PD) have also been conducted. All used AAV2 vectors but delivered different transgenes, and targeted different regions of the brain. A Phase I trial and subsequent Phase II trial using AAV2 encoding the glutamic acid decarboxylase (GAD) transgene, the rate-limiting enzyme for synthesis of γ-aminobutyric acid (GABA) (the major inhibitory neurotransmitter in the brain), was delivered to the subthalamic nucleus (99, 100). In the Phase I study, significant improvements in motor scores on the Unified Parkinson's Disease Rating Scale (UPDRS) were seen 3 months after gene therapy, and these persisted during 12 months of postsurgery monitoring (99). Patients also had a substantial reduction in thalamic metabolism, which is typically seen following effective interventions for PD (99). In the Phase II trial, the treated group showed a significantly greater improvement in UPDRS motor scores when compared with the control group during the 6-month course of the study (100). In another Phase I trial of treatments for PD, AAV encoding the dopaminergic neuroprotective factor neurturin, a naturally occurring structural and functional analogue of glial-cell-derived neurotrophic factor (GDNF), was administered to the putamen. The UPDRS motor score and time without troublesome dyskinesia were both improved 1 year following gene therapy administration (101), although the Phase II trial involving simultaneous delivery to the putamen and substantia nigra did not meet its primarily clinical end point. In two other Phase I trials, AAV2 encoding the transgene for L-amino acid decarboxylase (AADC), the enzyme that converts levodopa into dopamine, was delivered to the putamen (102, 103). Both studies demonstrated improvements in UPDRS motor scores and increases in AADC enzyme activity, which are promising early results for the treatment of this complex, prevalent disease. Additional studies involving AADC and GDNF are under way.

Following the success of using subretinal AAV2 to treat LCA2, investigators have initiated efforts to treat complex retinal diseases. Two clinical trials are under way that aim at enabling secretion of anti-VEGF (vascular endothelial growth factor) protein for treatment of wet age-related macular degeneration (wet AMD), following either a subretinal (clinicaltrials.gov identifier NCT01494805) or an intravitreal (clinicaltrials.gov identifier NCT01024998) injection of AAV2.

Other Gene Therapy Approaches

With a greater understanding of the immunological response to adenoviral vectors following the ornithine transcarbamylase deficiency trial (104) having been reached, efforts to develop adenoviral vectors have shifted toward applications that benefit from the strong host immune response, such as vaccines and oncolytic cancer therapy. Adenovirus vectors have been developed as vaccines against tuberculosis (105), malaria (106), HIV (107–109), and influenza (110, 111). Although the HIV vaccine demonstrated safety and an ability to induce T cell responses to HIV antigens

(107, 108), a follow-up efficacy study showed that the vaccine failed to protect against HIV infection. Furthermore, the incidence of HIV was actually higher in the treated groups than in the placebo groups, potentially because adenoviral vectors induce the expansion of T cells, which are the natural cellular hosts for HIV replication (109). Oral adenovirus vaccines for the H5N1 and H1N1 influenza viruses have shown promising results in preclinical studies (110) and a clinical trial, where the safety of the vectors was demonstrated, and antigen-specific T cell responses were elicited in patients receiving the vaccine (111). Adenovirus vectors expressing thymidine kinase have been evaluated in clinical trials for use alone or as combination therapies for several types of cancer (summarized in 112), and ONYX-015 (an oncolytic adenovirus engineered to selectively replicate in and lyse cancer cells lacking p53 expression) has demonstrated some efficacy in a clinical trial for treatment of advanced head and neck cancer (113).

Viral vectors based on vaccinia virus encoding granulocyte–macrophage colony–stimulating factor (GM-CSF) have also been employed in clinical trials to treat cancer. Vaccinia offers the considerable advantage over oncolytic adenoviruses and herpesviruses in that it is capable of being delivered and transmitted intravascularly, raising the possibility that a single administration could treat both the primary tumor and distant metastases. In a study of treatment for melanoma, 7 patients each received 12 increasing doses of the vaccinia virus vector JX-594 via intratumoral injections during 6 weeks (114). The two patients with the highest tumor burdens did not respond to treatment, but two patients had mixed responses, and two other patients became disease free (114). In a Phase I trial of JX-594 for treatment of refractory primary or metastatic liver cancer, 14 patients diagnosed with hepatocellular cancer, colorectal cancer, lung cancer, or melanoma who had solid tumor tissue in the liver were given 1–8 intratumoral injections of JX-594 encoding GM-CSF (71). Of the 10 patients who could be evaluated via radiography, 1 had progressive disease, 6 had stable disease, and 3 had a partial response (71). In a second clinical trial to test JX-594's ability to infect metastatic tumors, patients diagnosed with lung cancer, colorectal cancer, melanoma, ovarian cancer, thyroid cancer, pancreatic cancer, or gastric cancer were given JX-594 intravenously (69). Twelve of the 22 patients in this trial had stable disease at least 1 month following gene therapy, and 1 patient had a partial response (69). Of the 5 patients given the highest dose of JX-594, 4 had a partial response or stable disease (69). In a Phase II dose-escalation trial, 30 patients with advanced hepatocellular carcinoma received high or low doses of JX-594 via intratumoral infusion (70). The median survival for the patients increased to 6.7 months in the low-dose cohort and to 14.1 months in the high-dose cohort (70).

LESSONS LEARNED FROM CLINICAL STUDIES

Target Diseases

The first wave of efficacious gene therapy clinical trials have primarily focused on monogenic inherited disorders (9–11, 72, 79–84, 89), yet only 8.9% of clinical trials of gene therapy are for these indications (115). Although such diseases are rare, it has been increasingly recognized that they are highly promising clinical targets with well-defined genetic etiologies and tissue targets. Treatments for such indications are often eligible for orphan status and, thus, can qualify for market exclusivity in some countries as an incentive for developing new drugs.

Many targeted diseases have no therapies available, or where therapies exist for these indications, there are shortcomings. For example, ERTs for hemophilia A and B (diseases that affect more than 20,000 individuals in the United States), have been approved by the US Food and Drug Administration, although the annual costs of treatment are more than $100,000, and the high required frequency of administration (as many as 3 injections per week) can compromise quality of

life; however, longer-lived more stable forms of these proteins are beginning to receive regulatory approval. Similarly, for wet AMD, patients must receive a costly injection into their eye every 4–8 weeks, which places a high treatment burden on patients, physicians, and the healthcare system. Gene therapy offers the potential to reduce the lifelong financial burdens of such treatments while improving patients' quality of life. In cases where there are existing protein therapies, there are also the advantages of established clinical end points that can provide clarity on development and the regulatory approach.

Successes in treating monogenic diseases are reducing the risks of developing gene therapy for more complex disorders. For example, more than 64% of clinical trials of gene therapy have historically focused on cancer indications (115). Although cancer is an important disease due to its high prevalence, high mortality rate, and, thus, the high unmet medical need, it is a complex class of diseases that affect numerous genes, signaling pathways, and cell types in a heterogeneous manner across populations, and these issues have produced challenges for identifying initial targets for gene therapy. Many studies of gene therapy for cancer may have been conducted before gene therapy technology was sufficiently mature to tackle the complex nature of this class of disease. However, the remarkable progress in gene therapy involving T cell therapies or oncolytic viruses is generating momentum in this field (69–71, 75–78, 114), and these successes are now paving the way for continued improvements in gene therapies for cancer treatment.

Understanding Vector-Related Immunological Issues

To enhance the safety, efficacy, and broad utility of gene delivery vectors for all patients, vectors and administration protocols have had to contend with immunological issues. The most striking case of immune responses that can be elicited against viral-based gene therapy vectors is the fatality that occurred in the ornithine transcarbamylase deficiency trial due to the adenoviral vector. During this trial, a vector with the E1 and E4 units deleted—which still encoded and expressed numerous viral proteins—was administered to patients divided into six cohorts. Patients in the first five groups experienced mild toxicities—including fever, flu-like symptoms, and transient transaminitis—but no serious adverse events (14). The first patient in the highest-dose group experienced similar toxicity to the patients in the first five cohorts (14). However, the next patient treated with the vector developed an immune response that led to systemic inflammation, multiorgan failure, and eventually fatality (14). Based on numerous studies over the ensuing decade, researchers hypothesized that the patient's severe response was caused by adenoviral vector–induced activation of innate immune responses, specifically an acute release of inflammatory cytokines (14), although the reason underlying the heterogeneity in patients' immune responses to the vector was unclear. Regardless, this trial has strongly motivated researchers to develop vectors that are less immunogenic, particularly vectors that are completely deleted of viral genes, such as the AAV and lentiviral systems.

In addition to the obstacles associated with innate immune responses, gene therapy vectors delivered in vivo must overcome components of the adaptive immune system, including antibodies directed against vector proteins. Because most current vectors are based on human viruses, prior natural exposure to the viruses—which may occur early in life—can elicit antibodies that later neutralize the gene therapy vector. For example, a recent analysis found that neutralizing antibodies in serum against numerous AAV serotypes were present in a large percentage of the population, including against AAV2 (72%), AAV1 (67%), AAV9 (47%), AAV6 (46%), AAV5 (40%) and AAV8 (38%) (116). In a recent study in China, the seroprevalence was very high for AAV2 (97%) and AAV8 (82%), with considerable levels for AAV5 (40%) as well, and the fraction of the population that tested seropositive for AAV5 was also seropositive for AAV2 and AAV8 (117).

Antibodies in other bodily fluids have also been found in preclinical and clinical applications, including in human synovial fluid (118, 119), nonhuman primate cerebrospinal fluid (120), and nonhuman primate intravitreal fluid (121). Furthermore, these antibodies may be capable of cross-reacting with other AAV serotypes related to the ones tested. Although neutralizing antibodies against the AAV capsid do not appear to pose any safety risk to a patient, they have been associated with decreased therapeutic efficacy in several clinical trials. For example, in the first CUPID trial, the two patients who failed to respond following gene therapy had preexisting antibodies against the AAV1 capsid (96). Furthermore, in the second hemophilia trial, only patients with low levels of preexisting antibodies and who received intrahepatic administration of AAV2 showed vector transduction (55).

Antibodies can pose a challenge to future readministration of the same or a similar gene therapy vector even in patients who have low levels of preexisting antibodies prior to the administration of gene therapy. In a Phase I clinical trial of treatment for hemophilia B, anti-AAV antibody titers rose in all patients following intramuscular administration of AAV2 (88). In general, vector readministration could conceivably be needed in some cases, depending on the lifetime and replication rate of the transduced cells. Although readministration of AAV gene therapy for LCA2 was successful in the immune-privileged subretinal space (86), circulating antibodies in bodily fluids may prevent readministration in other tissues.

In addition to preexisting humoral immunity, cellular immunity against the AAV capsid can pose a challenge. For example, T cell responses against the AAV1 capsid were detected in half of the patients in the second clinical trial of alipogene tiparvovec, which potentially reduced its efficacy (80). In addition, in two clinical trials of treatment for hemophilia, transient transaminitis and loss of FIX expression were associated with the presence of AAV capsid-specific T cells (55, 89). These findings are potentially due to cross-presentation of capsid protein antigens on transduced cells, leading to $CD8^+$ T cell responses directed against these cells (55). One approach that may avoid such responses is transient immunosuppression (80, 81); in the AAV8 trial for hemophilia B, for example, transient administration of prednisolone at the onset of transaminitis suppressed cellular immune responses and enabled long-term FIX expression after the cessation of immunosuppression (55). An alternative is to avoid treating potential patients who have high antibody titers at baseline (55, 89), although this may preclude a substantial fraction of the human population.

Vaccinia virus gene therapy vectors appear to be less sensitive to immune responses involving antibodies. In two of the clinical trials of JX-594, no association could be found between the level of baseline antibodies and patients' outcomes (69, 70). All of the patients developed antivaccinia antibodies posttherapy, but these antibodies were not associated with transgene expression or patient outcomes (69, 70, 114).

FUTURE NEEDS

Vector Engineering

As gene therapy continues to mature and evolve toward a focus on disorders that are more difficult to treat, new technology will be needed. Although viral vectors used in successful clinical trials have been sufficient in those contexts, these and many preclinical studies have continued to reveal delivery challenges—including interactions with the immune system; poor transport to, and infectivity of, target cells; an incapacity to target delivery; and intracellular trafficking—that must be overcome to extend the promise of gene therapy to new and exciting applications. Because viruses were not evolved for our use in medical applications, many of the properties that enable these

viruses to survive in nature—including broad tissue tropism, random integration into the host cell genome, and lytic activity—are undesirable in the context of gene therapy. Furthermore, it is not surprising that many of the barriers established to prevent viral infections also pose challenges to using viral vectors for gene therapy. To address such concerns, next-generation retrovirus and lentivirus vectors are being explored that have more specific integration profiles (122) or mutations that cause the genome to be integration deficient (123). Furthermore, the engineering of AAV has focused on engineering the capsid to improve the transduction of cells in the presence of clinically relevant barriers to gene therapy (reviewed in 124).

Recent progress in AAV capsid engineering utilizes two approaches to create variants of the natural AAV serotypes that are improved over natural AAV for a given application, such as antibody evasion or efficient transduction of a therapeutically important cell type. Rational design combines knowledge of AAV biology and structural analyses to guide capsid changes, and directed evolution involves using iterative genetic diversification and selection to accumulate key genetic modifications that progressively improve a biomolecule's function. The latter approach can be successful even without knowledge of underlying structure–function relationships in the capsid proteins.

Rational design has been used successfully to improve several delivery properties of AAV. As one example, several strategies have been explored to enable AAV to overcome preexisting neutralizing antibodies (reviewed in 125). The discovery of linear and conformational AAV epitopes, and in silico structural analyses of the binding of anti-AAV antibodies to the AAV capsid, has been used to determine candidate positions that could be changed to reduce antibody binding. Subsequent mutagenesis of these regions of the capsid has led to the development of variants with somewhat reduced neutralization by human and mouse antibodies in vitro (126–128). Another method of decreasing neutralization by antibodies, developed through rational design, utilized empty AAV2-based capsid particles with mutated cell-receptor binding domains. These empty capsids were combined with gene therapy vectors to act as decoys to bind and block neutralizing antibodies that would otherwise bind to the gene therapy vectors (129). As another example, vectors with surface tyrosines can undergo phosphorylation, ubiquitination, and subsequent proteasomal degradation before trafficking to the nucleus. Vectors containing corresponding tyrosine-to-phenylalanine mutations are capable of transgene expression that is up to 10-fold higher in vitro and 30-fold higher in vivo in some applications (130, 131). In addition to reducing antibody neutralization and intracellular degradation, viral tropism has been altered through rational design. Structural alignment of AAV2 and AAV9 CAP protein sequences has been used to determine the amino acids necessary for galactose-receptor binding by AAV9, and those residues were subsequently grafted onto the AAV2 capsid. In murine liver, the resulting vector was capable of transducing cells using either heparan sulfate or galactose as the primary receptor and showed significantly higher infectivity of murine liver than AAV2 as well as higher preference for murine liver than AAV9 (132).

In many, or even most, situations, the structural basis for viral properties that must be changed is poorly understood. Directed evolution can be used as an alternative, powerful approach in the absence of complete knowledge of the AAV capsid to develop novel gene delivery vectors that address many current obstacles to gene therapy (**Figure 3**). For example, engineered capsids are being developed to reduce neutralization by anti-AAV antibodies. Early work that evolved vectors in the presence of neutralizing antibodies resulted in the generation of AAV2 variants with improved transduction in vitro (133, 134) and in vivo (134). More recent work aimed at creating broadly evasive AAV variants used multiple rounds of directed evolution in the presence of intravenous immunoglobulin (the pooled immunoglobulin G fraction from more than 1,000 patients); this yielded variants that withstood up to 35-fold higher concentrations of

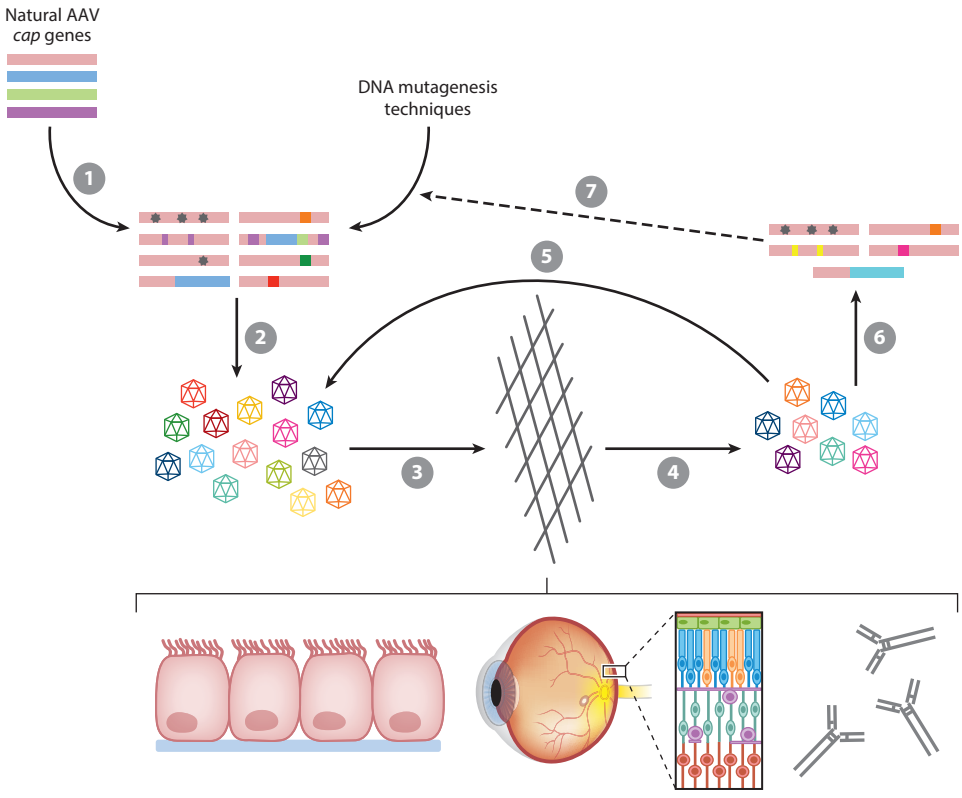

Figure 3

Schematic representation of directed evolution of adeno-associated virus (AAV). (❶) A viral library is created by mutating the *cap* gene using various DNA mutagenesis techniques. (❷) Mutant *cap* genes are used to package functional virus vectors such that the mutant capsid surrounds the corresponding *cap* gene containing the same mutations. (❸) The viral library is placed under selective pressure, for example, by transduction of human airway epithelium in vitro, transduction of retinal cells in vivo, or evasion of neutralizing antibodies. (❹) Successful viruses are recovered following selection. (❺) Successful clones are enriched through progressively more stringent selection steps. (❻) Viral DNA is isolated to uncover the sequences of successful clones. (❼) Selected *cap* genes can be further mutated to serve as new starting points to iteratively increase viral fitness.

intravenous immunoglobulin than AAV2 in human cells in vitro and also enhanced transduction in vivo in a passively immunized mouse model (135). These variants may facilitate the use of intravenous administration in patients with preexisting antibodies.

AAV capsids have also been evolved to more efficiently and specifically infect previously non-permissive cell types, including astrocytes, airway epithelial cells in the lungs, and cardiac and skeletal muscle cells (136–140). For example, following directed evolution, in vitro transduction of human airway epithelial cells was improved 100-fold over AAV2 (138); in vitro transduction of neural stem cells was enhanced 50-fold over AAV2 (141); and in vitro transduction of human pluripotent stem cells was improved 3-fold over AAV2 (142). In vivo–directed evolution systems are also being harnessed to develop engineered vectors for which no adequate in vitro models exist. AAV variants have been developed that are capable of increased murine heart preference compared with AAV9 (139), crossing a seizure-compromised blood–brain barrier (143), and preferentially transducing human hepatocytes in a mouse xenograft model via intravenous injection (144).

Vectors have been developed to improve the transduction of retinal cells following intravitreal injection as part of an effort to extend the success of the LCA2 clinical trials. Natural AAV serotypes are incapable of penetrating from the vitreous fluid of the eye to the back of the retina, where the photoreceptors and retinal pigment epithelial cells impacted by retinal disease reside. To overcome this barrier, subretinal administration involving injection of the vector underneath the retina was required; however, this is an invasive approach that is accompanied by transient retinal detachment and is problematic for some types of retinal degeneration. Several studies have evolved novel AAV vectors to address this challenge. First, an AAV variant developed for improved glial cell transduction was capable of highly specific (94%) and efficient transduction of Müller glia—a cell type that extends all the way from the retinal surface to photoreceptors—in the rat retina following intravitreal injection (136). In a rat model of retinitis pigmentosa, Müller glia transduced by the vector secreted high levels of a neuroprotective factor that slowed retinal degeneration (136). In a subsequent study, in vivo directed evolution was utilized to engineer a vector capable of enhanced tissue penetration to access cell types that were permissive to infection, specifically an AAV variant that could directly infect photoreceptors following intravitreal injection (145). The resulting vector, which mediated high levels of retinal gene expression in mouse models and moderate levels in nonhuman primates, was also capable of rescuing the wild-type phenotype in mouse models of X-linked retinoschisis and LCA2 (145).

Although most engineered vectors are still being investigated in preclinical models, a recent Phase I clinical trial for treatment of Duchenne muscular dystrophy using a chimeric AAV2/AAV1 vector demonstrated that mutated AAV vectors can be safe and well tolerated (90), paving the way for future clinical trials using engineered AAV capsids.

Cargo Engineering

In conjunction with improving vectors, making enhancements to the genetic material being delivered may improve the safety and efficacy of gene therapy. For example, the use of cell-type-specific promoters instead of strong viral promoters may reduce the risk of activating neighboring endogenous genes for an integrating vector such as lentivirus (12). Cellular promoters also offer the potential of targeting transcription to only the cell or tissue of interest, and, thus, reducing the side effects that could result from off-target gene expression or immune presentation of therapeutic protein antigens. Alternatively, cell-specific microRNA target regions have been engineered into the transgene cassette. These regions are bound by microRNA in cells or tissues that express these microRNAs, thereby specifically downregulating transgene expression in off-target cells (146). Furthermore, therapeutic transgenes can be codon-optimized for expression in human cells to yield higher gene expression (89). In addition to increasing efficacy, improving both genetic cargoes and delivery vectors could result in a need for lower doses of the gene therapy product, which would lower the manufacturing burden and potentially reduce cellular immune responses to vector proteins.

In addition to delivering exogenous transgenes, recent advances in site-specific DNA nucleases have enabled targeted editing of endogenous genes. Such nucleases can mediate either the targeted knockout of a pathological allele to treat dominant disease or even the editing and repair of a mutated allele to treat a recessive disorder. As a prime example of such precision medicine, zinc-finger nucleases (ZFNs) combine a DNA nuclease with engineered zinc fingers that bind to a specific DNA sequence in order to introduce double-stranded breaks at target sites in a cellular genome. ZFNs designed to disrupt the gene encoding CCR5—which serves as a coreceptor for HIV infection of $CD4^+$ T cells—in HSCs have been explored as a gene therapy option to treat HIV (147). Results reported from one study observed that five of the eight participants had a

declining trend in HIV DNA at 6 months posttherapy and an increase in mean $CD4^+$ T cell counts (148). Another study reported that the CCR5-disrupted HSCs resulted in an increase in $CD4^+$ T cell counts that peaked at 7–14 days posttherapy, and 5 of the 9 patients maintained $CD4^+$ T cell counts above 500 cells/mm^3 for 1 year posttherapy (149).

Site-specific endonucleases can also be used to increase the efficiency of homologous recombination to mediate endogenous gene repair. A preclinical study in a mouse model of hemophilia B used an AAV8 vector encoding a ZFN targeted to the *F9* gene (encoding FIX); this induced double-stranded breakage in the genome, and mediated homologous recombination (150) with a codelivered, promoterless FIX cDNA fragment (151). This strategy produced a level of gene correction sufficient to improve blood clotting times (151) and demonstrated that fragments of large cDNAs could be used to repair focal mutations within large genes. In addition to ZFNs, the use of transcription-activator-like effector nucleases (TALENs) and RNA-guided engineered nucleases that are based on the CRISPR (clustered regularly interspaced short palindromic repeat)–Cas (CRISPR-associated protein) system (reviewed in 152) are highly promising technologies being explored in preclinical applications of precise genome editing.

SUMMARY

Initial setbacks in the pioneering research into adenovirus and retrovirus vectors led to a better understanding of the vector- and integration-related toxicities that needed to be overcome for viral gene therapy to become a safer and more effective therapeutic modality (12–14). Thus, the lessons learned from the early studies and decades of research into new vectors, particularly AAV and lentivirus, have enabled the first clinically promising gene therapies. Clinical trials utilizing lentivirus-based vectors have demonstrated efficacy for treating SCID, X-linked adrenoleukodystrophy, Wiskott–Aldrich syndrome, and leukemia (9, 11, 72, 73, 75–78). Furthermore, AAV-based gene therapy has been successful in clinical trials of treatments for LCA2, choroideremia, and hemophilia B, and an AAV-based vector has been approved in Europe to treat LPL (55, 79–89).

Although the current vectors have been sufficient to achieve some clinical successes, many clinical trials have continued to reveal a number of delivery and cargo challenges. Improvements to vector systems, made by using directed evolution and rational design approaches, promise to create next-generation vectors capable of overcoming many of the gene delivery challenges that currently limit clinical efficacy. Furthermore, improvements in the cargo will include the development of short promoters and microRNA-binding elements that mediate cell-specific gene expression to reduce potential off-target effects, as well as targeted DNA nucleases for ablation or repair of endogenous genes. Moreover, newer generations of oncolytic viruses will further broaden the promise of harnessing viruses to treat cancer. Collectively, these innovations and advances will extend the reach of viral therapeutics to treat a broader range of human diseases and, thus, address serious unmet medical needs.

DISCLOSURE STATEMENT

M.A.K. is an inventor on a patent related to viral vector engineering. T.W.C. is an employee, director, and officer of Avalanche Biotechnologies, a company focused on clinical development of gene therapy. D.V.S. is an inventor on several patents related to viral vector engineering and cofounder of a company focused on clinical development of viral vectors for gene therapy; he also serves on the supervisory board of another company focused on clinical development in gene therapy. D.V.S. is the primary investigator for National Institutes of Health grants that fund gene therapy research.

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