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Entering the Modern Era of Gene Therapy

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Abstract

Gene therapies are gaining momentum as promising early successes in clinical studies accumulate and examples of regulatory approval for licensing increase. Investigators are advancing with cautious optimism that effective, durable, and safe therapies will provide benefit to patients—not only those with single-gene disorders but those with complex acquired diseases as well. While the strategies being translated from the lab to the clinic are numerous, this review focuses on the clinical research that has forged the gene therapy field as it currently stands.

INTRODUCTION

Gene therapy is the transfer of genetic material to a patient to treat a disease. Although the concept has existed for many decades, clinical investigation began in 1990, when the first clinical study, for a rare immunodeficiency disorder, was undertaken at the US National Institutes of Health. Since then, more than 2,500 clinical studies have been initiated for a broad range of applications, from a variety of monogenic diseases to infectious diseases, complex neurodegenerative disorders, and cancer (**Figure 1**).

Methods of classifying gene therapy include sorting by the class of disease (genetic disease versus complex acquired disorder), by the characteristics of the gene delivery vehicle (integrating versus nonintegrating), and by whether the vector is administered in vivo (directly into the patient) or ex vivo (in cultured cells taken from the patient that are subsequently transplanted back).

In its most straightforward incarnation, the goal of gene therapy for genetic diseases is long-term expression of the transferred gene at levels high enough to be therapeutic, an approach sometimes called augmentation gene therapy. The transferred gene is most frequently a normal copy of a mutated gene. One can also suppress expression of a detrimental gene by employing RNA interference or genome editing tools. And it is theoretically possible, though not yet in clinical trials, to use genome editing techniques to correct a mutated gene in its precise genomic location through homologous recombination with a donor template or via base editing (2) (**Figure 2**).

One of the major differentiating factors for in vivo versus ex vivo gene therapy is the choice of vectors (**Table 1**). As noted above, vectors can be divided into predominantly integrating and predominantly nonintegrating. When introducing genetic material into stem cells, it is critical to use integrating vectors so that the donated DNA will be incorporated into the genome of the stem cell, will be replicated when the cell undergoes division, and thus will be passed to all daughter cells. For in vivo gene therapy, one is often targeting long-lived postmitotic cells. In these cells, since they are no longer undergoing division, one can achieve long-term expression as long as the transferred DNA is stabilized in the cell; episomal stabilization is adequate to drive expression for the cell's life span. Most gene therapy strategies for treating genetic disease are now coalescing around two kinds of vector: lentiviral vectors for ex vivo gene transfer into hematopoietic and other stem cells (3) and adeno-associated viral (AAV) vectors for in vivo gene transfer into postmitotic cell types (4).

Before any experience with clinical gene therapy, investigators had theorized about its potential risks as a treatment for genetic diseases (**Table 2**). As clinical experience accumulated through the 1990s, it became apparent that the risks sorted into two main groups. The early integrating vectors

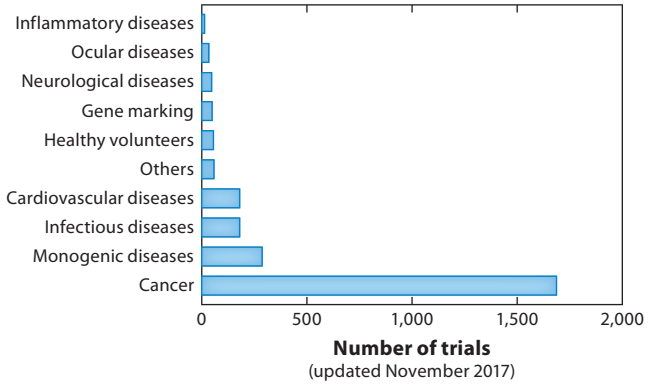

Figure 1

Indications in gene therapy clinical trials. The bar graph classifies clinical gene transfer studies by disease. Adapted from Reference 1.

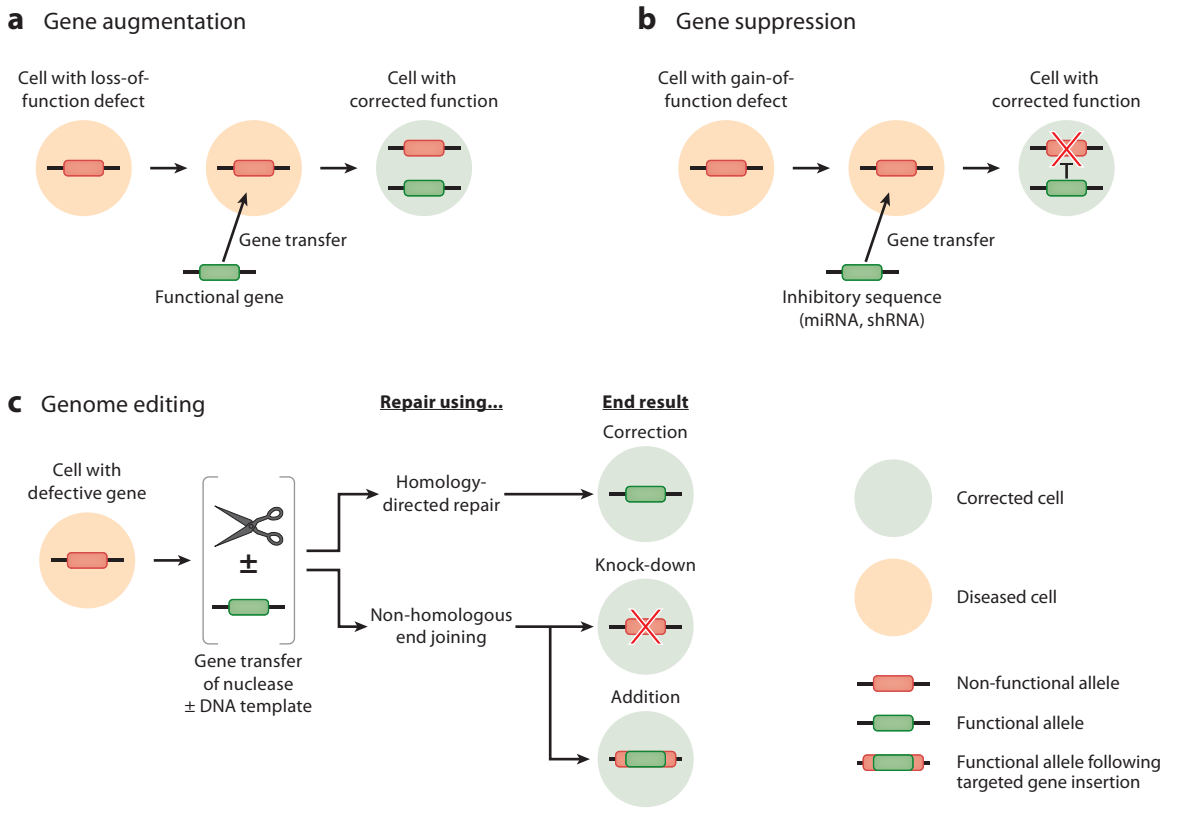

Figure 2

(*a*) The goal of gene augmentation is to restore normal cellular function by providing a functional copy of a gene *in trans* (i.e., without affecting the diseased gene itself, which will still remain in the cell). Examples of this approach include the in vivo treatment of diseases such as Leber congenital amaurosis, the hemophilias, and spinal muscular atrophy (model examples of ocular, liver, and central nervous system indications, respectively) and the ex vivo treatment of primary immunodeficiencies such as X-linked severe combined immunodeficiency. (*b*) In some other indications, such as Huntington's disease, cellular function is lost as a result of toxic accumulation of a defective protein. Gene suppression aims at restoring cellular fitness by reducing the expression of the mutated gene via RNA interference. (*c*) While genome editing does not necessarily require the use of a nuclease, the efficiency of gene-specific editing in mammalian cells is typically enhanced by the induction of a DNA double-strand break at the target site. The choice of one DNA repair mechanism over another will determine the outcome of genome editing. In its simplest form, after DNA cleavage occurs, the break is rejoined by non-homologous end joining, which may result in gene knock-down if repair is imperfect. In the presence of an exogenous template coding for a functional gene, DNA repair may also result in the in situ correction of the mutated gene via homologous recombination. A third potential outcome is the insertion of the DNA template via non-homologous end joining, which will result in gene addition rather than correction.

carried the risk of insertional mutagenesis, borne out by the development of T cell leukemia in children treated for X-linked severe combined immunodeficiency (SCID) (5); and for in vivo vector administration, the risk was related to deleterious immune responses. For some vectors, these could be life threatening (6), while for others they represented a risk to long-lasting efficacy but not safety (7). For cancer gene therapies, risks are often associated with excessive T cell activation.

Gene therapy's first success stories involved ex vivo applications using hematopoietic stem cell (HSC) transplantation of autologous gene-corrected cells for the treatment of primary immunodeficiencies. In vivo gene therapy lagged behind, but the prospect of success appeared to be

Table 1 Viral vectors discussed in this review

Features	Retroviral	Lentiviral	Adenoviral	AAV
Viral genome	RNA	RNA	DNA	DNA
Cell division requirement for target cell	Yes	G1 phase	No	No
Packaging limitation	8 kb	8 kb	8–30 kb	5 kb
Immune responses to vector	Few	Few	Extensive	Few
Genome integration	Yes	Yes	Poor	Poor
Long-term expression	Yes	Yes	No	Yes
Main advantages	Persistent gene transfer in dividing cells	Persistent gene transfer in transduced tissues	Highly effective in transducing various tissues	Elicits few inflammatory responses, nonpathogenic

Abbreviation: AAV, adeno-associated viral.

at hand by the turn of the millennium. However, several high-profile adverse events, involving malignant transformation due to insertional mutagenesis with integrating vectors and the death of a patient in an adenoviral vector trial, affected the willingness of physicians and patients to participate in trials and the willingness of investors to back further work in the field. Gene therapy's resurgence after approximately a decade was largely due to successes in trials that utilized both ex vivo strategies (for X-linked SCID, adenosine deaminase deficiency, and adrenoleukodystrophy) and in vivo approaches (Leber congenital amaurosis type 2 and hemophilia B).

Today, six gene therapies have received approval in the Western world. Glybera®, an AAV-based therapy for familial lipoprotein lipase deficiency, received conditional marketing approval from the European Medicines Agency (EMA) in 2012. IMLYGIC®, a genetically modified herpes simplex virus type 1 for the local treatment of unresectable lesions in patients with melanoma, was approved by the US Food and Drug Administration (FDA) in 2015. Strimvelis®, a γ-retrovirus-based therapy for the treatment of severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID), gained EMA approval in 2016. Three gene therapies were FDA

Table 2 Potential complications of clinical gene therapy

Potential complications of gene therapy	Strategies to mitigate risks
Gene silencing—repression of promoter	Use endogenous cellular promoters, avoid viral-derived regulatory sequences
Genotoxicity—complications arising from insertional mutagenesis	Use vectors with safer integration profile (e.g., self-inactivating lentiviral vectors) Sequence-specific integration (i.e., genome editing)
Phenotoxicity—complications arising from overexpression or ectopic expression of the transgene	Control transgene expression spatially (e.g., endogenous, tissue-specific promoters) and temporally (on/off switch)
Immunotoxicity—harmful immune response to either the vector or transgene	Carefully monitor T cell reactivity to the vector and transgene to initiate immune suppression if needed
Risk of horizontal transmission[a]—shedding of infectious vector into the environment	Monitor vector shedding in preclinical models when developing novel vectors
Risk of vertical transmission—germline transmission of donated DNA	Use of barrier contraceptive methods until vector shedding is negative

[a]Clinical experience with the four vectors described above shows that the risk of horizontal transmission is very low.

approved in 2017. KYMRIAH® and YESCARTA® are CD19-directed genetically modified autologous CAR T cell immunotherapies; both are indicated for the treatment of non-Hodgkin lymphoma, and KYMRIAH® is also indicated for the treatment of acute lymphoblastic leukemia. LUXTURNA® is an AAV-based gene therapy indicated for the treatment of biallelic *RPE65* mutation-associated retinal dystrophy.

The goal of this review is to provide a framework for assessing clinical results from ongoing studies, as the numbers of reports continue to increase. With several product candidates now undergoing regulatory review, it appears likely that clinicians will have increasing opportunities to generate their own assessments of gene therapy as a treatment modality.

EX VIVO GENE THERAPY

Gene Therapy in Hematopoietic Stem Cells

Ex vivo gene therapy approaches to treat genetic diseases have mainly targeted HSCs, the self-renewing cells that give rise to all the blood cell lineages. Due to the multiple cell divisions that these cells undergo, either to self-renew or to differentiate into a particular cell type, the therapeutic gene needs to be stably integrated into the host genome to be present in all daughter cells.

In the late 1990s, a clinical trial by Fischer and colleagues (8) provided the first convincing demonstration of therapeutic effect of gene transfer for the treatment of X-linked SCID. In SCID-X1, an autosomal recessive disorder, disease is due to mutations in the gene encoding the γc subunit of the cytokine receptor. This results in an absence of circulating T and natural killer (NK) cells and poorly functioning B lymphocytes, rendering the disease fatal in the early years of life unless successful bone marrow transplantation reconstitutes the immune system. In the original SCID-X1 clinical studies, based on first-generation γ-retroviral vectors in which the gene was expressed under the control of viral regulatory elements, the therapy showed promising signs of efficacy, with successful correction of the T cell defect and partial restoration of the NK and B cell lineages (8). Complete reconstitution of the immune system, including documented responses to standard childhood vaccinations, clearing of infections, and remarkable gains in growth, occurred in most of the treated children. However, 5 of 20 patients eventually developed T cell leukemia in two separate trials as a result of vector insertional oncogenesis (5, 9).

At nearly the same time, a group in Milan was demonstrating remarkable success in trials for a related rare immunodeficiency disorder, ADA-SCID (10). Caused by mutations in the adenosine deaminase (ADA) gene, the condition is characterized by the absence of cellular and humoral immune function, organ damage caused by the accumulation of toxic metabolites, and, typically, a fatal outcome very early in life. Although initial studies failed to generate a therapeutic effect, subsequent trials that incorporated innovations designed to provide a survival advantage to the cells transduced with a retroviral vector containing the ADA gene (by discontinuing enzyme replacement at the time of reinfusion of the transduced cells), and to promote engraftment (by administration of a mild conditioning regimen prior to administering the gene-modified cells), met with clear therapeutic success in two patients in the early 2000s (10). In 2009, a report on the long-term outcome of these two children and results in eight additional patients showed that HSC gene therapy was a safe, effective, and durable treatment for SCID in patients with ADA deficiency, with clear disease-modifying benefits (11). In 2016 this treatment received EMA approval, a landmark event for the field. For patients who have a human leukocyte antigen (HLA)-identical sibling, bone marrow transplantation is still the first-line therapy, but most patients do not. For those without an HLA-identical match, gene therapy has comparable efficacy to enzyme replacement,

does not require repetitive injections, and does not run the risk of neutralizing antibodies to the bovine enzyme (12).

Interestingly, the insertional mutagenesis events that characterized the X-linked SCID trial were never observed in the ADA-SCID trial, even though detailed studies of integration sites revealed little difference in sites of genomic integration in the two trials (13–15). This led to speculation that other factors, including the transgene product itself, may have contributed to the outcome (16). The SCID trials supported the hypothesis that gene transfer into HSCs could be used to treat any genetic disease for which allogeneic bone marrow transplantation was therapeutic. Moreover, the use of genetically modified autologous cells carried several advantages: no risk of graft-versus-host disease, guaranteed availability of a "donor" (unless the disease itself damages the stem cell population of the patient), and low likelihood of failure of engraftment.

Similar to SCID-X1, clinical experience with γ-retroviruses resulted in several cases of malignant transformation related to insertional oncogenesis in patients suffering from Wiskott-Aldrich syndrome (17) and X-linked chronic granulomatous disease (18). Based on studies in tumor-prone animal models (19) and the clinical data available, lentiviral vectors, another type of integrating vector, are expected to be less genotoxic than retroviral vectors. This is in part due to differences in integration patterns, as γ-retroviral vectors tend to integrate near the start of transcriptional units whereas lentiviral vectors tend to integrate randomly in the transcriptional unit but not upstream of the transcriptional start (20). Importantly, lentiviral vectors under clinical development have all viral regulatory elements deleted. These two characteristics confer lower risk for activation of adjacent genes.

Ex vivo approaches using lentiviral vectors can establish long-term, stable transgene expression in the central nervous system (CNS). Cartier et al. (21) conducted the first trial of lentiviral vector transduction of HSCs for a neurodegenerative disorder, X-linked adrenoleukodystrophy (ALD). X-linked ALD is a fatal demyelinating disease of the CNS caused by mutations in the gene encoding an adenosine triphosphate–binding cassette transporter (*ABCD1*). Following lentiviral transduction of autologous HSCs, dramatic stabilization of disease occurred, demonstrating that stem cell transduction could work for neurodegenerative as well as immunologic disorders (21). This corrective effect is based on expression of the donated gene in the microglia, which arise from circulating monocytes that traffic to the brain. Investigators carried this observation one step further to develop a treatment for metachromatic leukodystrophy, another neurodegenerative disorder that had previously responded poorly to bone marrow transplantation. Metachromatic leukodystrophy is a lysosomal storage disorder caused by mutations in the gene encoding arylsulfatase A (*ARSA*). The late infantile form of the disease is characterized by progressive motor and cognitive impairment, followed by death within a few years of onset due to accumulation of the ARSA substrate sulfatide in oligodendrocytes, microglia, and some neurons. Transduction of autologous HSCs from children born with the disease, performed when they were presymptomatic, led to preservation and continued acquisition of motor and cognitive milestones as long as 32 months after affected siblings had begun to miss milestones (22).

The β-hemoglobinopathies require more extensive transduction of HSCs to achieve a therapeutic effect. They are the most common monogenic disorders, with β-thalassemia and sickle cell disease (SCD) showing the highest prevalence (23). Given the scarcity of HLA-identical donors, development of successful gene therapies to correct autologous HSCs would represent a major breakthrough that could benefit large numbers of patients worldwide. One of the main challenges of gene therapy for β-hemoglobinopathies is the fact that gene-corrected cells have no selective advantage over diseased cells. Additionally, functional globin needs to be expressed in a lineage-specific manner and in substantial amounts to exert a therapeutic effect (reviewed in 24). β-Thalassemia is caused by loss-of-function mutations in the β-globin gene, resulting in

hemolytic anemia. Depending on its severity, the disease is classified as minor, intermediate, or major. Patients with β-thalassemia major require constant blood transfusions to treat the severe anemia and iron chelation to prevent the resulting excess iron. SCD, in contrast, is caused by an amino acid substitution that generates a defective β-globin peptide, which in turn results in rigid, sickle-shaped red blood cells that obstruct microvessels and have a shortened half-life. Despite significant treatment advances, SCD remains a chronic disease with significant morbidity and reduced life expectancy, even in developed countries (25).

The first successful gene therapy approach in an adult with β-thalassemia was reported in 2010 (26). Building on previous observations, myeloablative conditioning preceded reinfusion of the lentiviral-transduced autologous HSCs, resulting in stable hemoglobin production and transfusion independence for 33 months. Of note, most of the therapeutic benefit resulted from a dominant cell clone, in which the integrated vector caused transcriptional activation of *HMGA2*. This benign clonal dominance declined after several years (24). Industry- and academic-sponsored clinical trials using β-globin lentiviral vectors are now open at multiple sites and in multiple countries (NCT03276455, NCT03207009, NCT01639690, NCT02453477, NCT02151526). A recent report described transfusion independence following gene transfer in 12 of 13 β-thalassemia patients with some functional β-globin (i.e., non-β^0/β^0 genotypes). Where β-globin expression was completely absent (i.e., β^0/β^0 genotype), median annualized transfusion volume was significantly decreased, but RBC transfusions could be discontinued in only 3 of 9 patients (27). Of note, no serious adverse events related to the drug product and no clonal dominance related to vector integration were observed in this trial.

A single report exists on the use of a lentiviral approach to treat SCD (28). HSCs from a 13-year-old patient were treated with a lentiviral vector carrying a modified β-globin gene encoding an antisickling variant [βA87Thr:Gln (βA-T87Q)] and transplanted back following myeloablation. Fifteen months after treatment, the level of therapeutic antisickling β-globin remained at ∼50% of β-like-globin chains, which prevented the recurrence of sickle crises and corrected the biologic hallmarks of the disease. Larger cohorts of patients and longer follow-up are needed to determine whether this approach can be more broadly applied.

After two decades of follow-up since the first patients were treated with cells modified using integrating vectors, the overall results from clinical studies support the superior safety of lentiviral over γ-retroviral vectors. These approaches, however, require complex procedures and trained personnel to harvest, transduce, and reinfuse the hematopoietic target cells, and the design and manufacture of the lentiviral vectors are also challenging. All these factors pose a limit to the number of centers that can make these therapies available to patients. For example, Strimvelis® (therapy for ADA-SCID) is currently offered at a single center in Milan.

Gene Therapy for the Treatment of Cancer

The majority of clinical gene transfer experience has been in subjects with cancer (**Figure 1**). In contrast to trials for genetic disease, where the choice of the transgene is straightforward, gene therapy trials for cancer have utilized an array of strategies to target the tumor. Some of the earliest cancer gene therapy trials focused on local delivery of a prodrug or a suicide gene that would increase the sensitivity of tumor cells to cytotoxic drugs. A frequently used strategy has been intratumoral injection of an adenoviral vector expressing the thymidine kinase gene (*TK*). Cells that take up and express TK can be killed after the administration of ganciclovir, which is phosphorylated to a toxic nucleoside by TK (29). A similar approach includes the injection of an adenoviral vector that expresses cellular tumor antigen p53 (Ad-p53), which induces growth arrest and apoptosis in tumor cells. Ad-p53 was the first gene therapy to be approved, although it is not

available in the United States or Europe. These early studies established proof-of-principle for cancer gene therapy but clearly indicated that targeting a sufficient number of cells, even when the vector could be injected into the tumors directly and repeatedly, represented a serious obstacle to achieving full efficacy (30).

Another approach uses oncolytic viruses (OVs) that selectively replicate in tumor cells without harming normal cells. The replicating virus can proliferate within the tumor, facilitating eventual tumor clearance. We do not discuss OVs in detail, but recent clinical trials with native and genetically modified viruses have reported a favorable risk–benefit ratio, with meaningful clinical benefit to patients (reviewed in 31). Talimogene laherparepvec (IMLYGIC®), a modified oncolytic herpesvirus encoding granulocyte-macrophage colony-stimulating factor, was approved for melanoma treatment in 2015, representing the first OV to gain approval by the FDA as an anticancer therapy in the United States (32).

Since metastasis, rather than uncontrolled growth of the primary tumor, is the source of mortality for most cancers, systemic gene therapy is of considerable interest. Genetically modified immune cells expressing either chimeric antigen receptors (CARs) or T cell receptors (TCRs) isolated from tumor-reactive T cells represent a new class of therapeutics that has shown encouraging success for the treatment of some types of cancer. CARs endow the gene-modified cell, typically a T cell, with novel specificity to target antigens expressed on the surface of tumor cells. A CAR is usually formed by linking a single-chain variable fragment derived from an antibody to intracellular signaling chains such as the TCR-derived CD3ζ domain and one or more intracellular costimulatory domains. This architecture allows circumvention of the need for HLA recognition, thus expanding the physiologic T cell repertoire, for instance, to self-antigens expressed in transformed cells. On the other hand, TCR-engineered T cells can recognize intracellular antigens not expressed on the surface of tumor cells as long as they are HLA-presented. Both treatment modalities involve isolation of T cells from the patient through apheresis, gene transfer (usually via retro- or lentiviral vectors), ex vivo T cell expansion, and host conditioning before cells are transferred back into the host (**Figure 3**).

Therapies targeting CD19, an antigen present in most B cell malignancies but absent in normal tissues other than the B cell lineage, are at the forefront of CAR-based technology. A turning point occurred in 2010 and 2011 when the positive outcomes from three CAR therapy trials were published (33–35). These studies showed unprecedented antitumor activity in patients with B cell lymphoma (36, 37), chronic lymphocytic leukemia (NCT01029366, NCT00466531), or B cell acute lymphoblastic leukemia (38). In less than 10 years, CAR T cell–based immunotherapy has transitioned from preclinical proof-of-concept to several successful trials for B cell malignancies, including multiple myeloma (NCT02135406) (39). This success culminated with the recent FDA and EMA approval of two CAR T therapies targeting CD19-expressing B cells. As mentioned in the Introduction, both KYMRIAH® and YESCARTA® are indicated for the treatment of adult patients with relapsed or refractory large B cell lymphoma, and KYMRIAH® is also indicated for the treatment of relapsed and refractory B cell acute lymphoblastic leukemia in pediatric and young adult patients. While CD19-targeting CARs remain the benchmark for all other CAR treatments, CAR research has expanded beyond CD19 and includes other hematologic malignancies (reviewed in 40) and treatment of solid tumors (41).

The first clinical trial using TCR-engineered T cells was reported in 2006 and described objective regression of metastatic melanoma lesions in 2 of 17 patients following adoptive cell transfer of MART-1-targeted lymphocytes (42), paving the way for a series of studies targeting this and other melanoma antigens. While TCR-modified cells have been used to treat different cancer types (reviewed in 43), perhaps the most encouraging clinical outcomes have been observed for multiple myeloma, with response rates as high as 80% (44).

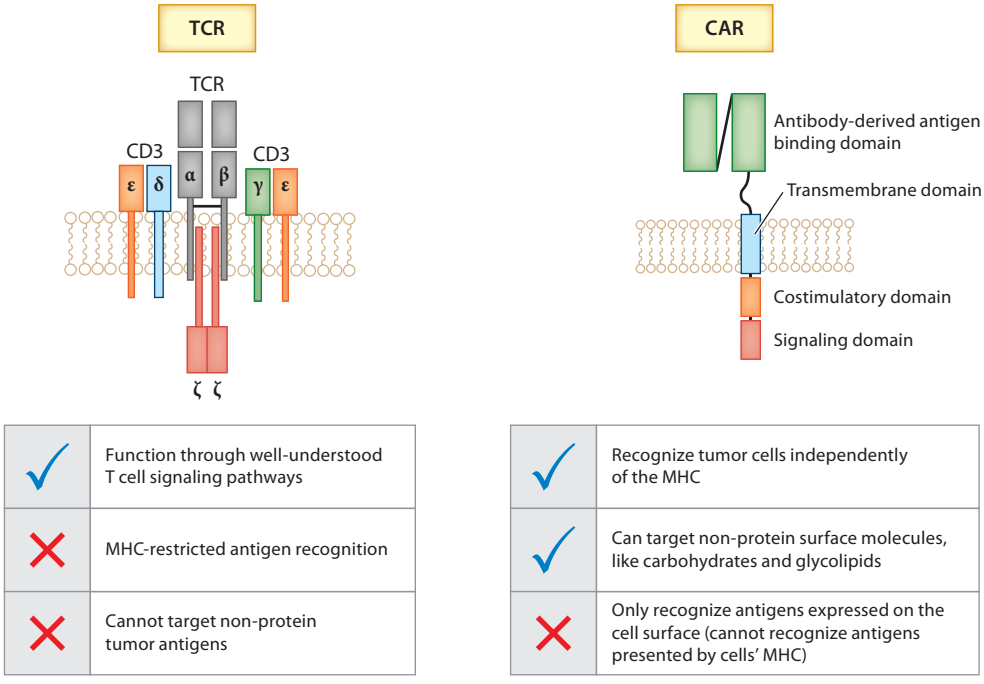

Figure 3

Genetic engineering of T cells. TCRs have the ability to recognize any protein expressed by a cell and can potentially distinguish healthy cells from tumor cells through the specific recognition of mutant protein variants expressed solely in the latter. MHC-restriction and the inability to target non-protein antigens are two of their main limitations. CARs recognize antigens independently of the MHC and can target non-protein surface molecules, like carbohydrates and glycolipids. However, CARs can only recognize antigens expressed on the cell surface. The lack of cancer-specific targets is an additional limitation for CARs. Abbreviations: CAR, chimeric antigen receptor; CD3, cluster of differentiation 3; MHC, major histocompatibility complex; TCR, T cell receptor.

Unfortunately, these promising therapies are not risk free. To date, the most prevalent adverse effects following infusion of CAR T cells result from on-target T cell activation, including cytokine release syndrome, macrophage activation syndrome, and tumor lysis syndrome (45). These toxicities are often short-lived and can be addressed by supportive care. Serious adverse events, some fatal, have also been observed in trials with engineered T cells, often related to off-target effects (46) or on-target, off-tumor toxicity (47). Controlling the duration of exposure and introducing a safety off-switch may decrease the risk–benefit ratio. Although the clinical responses in some specific tumors are revolutionary, more studies are needed to better understand the mechanisms behind, and achieve better control of, adverse reactions.

Despite their current limitations, such as relapses due to loss of expression of the CAR-targeted antigen (48), gene therapies for the treatment of cancer (as either single or multimodal regimens) seem to be ready to converge toward a new generation of therapeutics to improve outcomes for cancer patients (49).

IN VIVO GENE THERAPY

Retinal Gene Therapy

AAV-mediated gene transfer for inherited retinal dystrophies has provided a potential treatment for a category of diseases that had lacked any pharmacologic therapy. Gene transfer for autosomal

recessive mutations in the gene encoding retinal pigment epithelium-associated 65-kDa protein (RPE65) represented arguably the first clinical proof-of-concept of any in vivo gene therapy. Three independent trials (NCT00481546, NCT00516477, and NCT00643747) demonstrated improved visual function in several young adult patients in the initial months after a single unilateral subretinal administration of AAV2 (50–52). However, only one of these studies progressed to injection of the contralateral eye and a phase III study (53, 54). The other studies subsequently reported that the initial clinical benefit gradually declined and was lost 1–3 years following AAV administration in some or all of the subjects studied (NCT00481546 and NCT00643747) (55, 56). This phenomenon was not observed in the trial that progressed to phase III (54). All three studies utilized the same AAV2 capsid serotype and a single-stranded genome configuration of the RPE65 cDNA. The reasons behind the differences in their outcomes are debated but likely reflect differences in design of the expression cassette, final formulation of vector, surgical procedure, and perioperative immunomodulatory regimen. These variables may affect the amount of vector delivered to target cells, the type and/or magnitude of a potential immune response, degree of transgene silencing, or other parameters that affect clinical outcome. The hypothesis that subjects at an earlier stage of disease would have a larger population of target cells that could support the activity of the donated gene prompted Maguire et al. (57) to expand the scope of their phase I trial to include children. After encouraging results from these studies, the authors went on to assess whether functional vision could be further improved by delivering AAV2-RPE65 to the contralateral, formerly better-seeing eye (NCT01208389) (53, 58). Overall, improvements in efficacy outcomes were observed, without significant immunogenicity, laying the groundwork to progress to a phase III clinical trial (NCT00999609) (54). The randomized controlled phase III study showed superior performance of the intervention group over the control group for the primary and the first two secondary endpoints, as well as a trend toward improvement for the third secondary endpoint (54). These efforts culminated in the FDA approval of LUXTURNA® in 2017 for the treatment of patients with biallelic *RPE65* mutation-associated retinal dystrophy, representing the first gene therapy for a genetic disease approved in the United States.

The early successes with gene therapy due to autosomal recessive mutations in *RPE65* fueled additional trials for other inherited retinal dystrophies. Trials using AAV vectors are currently under way for Leber's hereditary optic neuropathy (NCT02161380, NCT02652767), choroideremia (NCT02341807, NCT02407678), X-linked retinoschisis (NCT02317887, NCT02416622), X-linked retinitis pigmentosa (NCT03252847), and achromatopsia (NCT02935517, NCT02599922, NCT03001310). In addition, trials utilizing lentiviral vectors to target photoreceptors are under way for Stargardt's disease (NCT01367444) and Usher syndrome type 1B (NCT01505062). In addition to genetic disease, gene therapy approaches have been used on an investigational basis for age-related macular degeneration (NCT01024998, NCT01301443, NCT01494805, NCT03066258).

Liver-Directed Gene Therapy

Much of the clinical experience in liver-directed gene therapy comes from trials for the treatment of hemophilia B using AAV vectors. Hemophilia is the X-linked bleeding diathesis caused by mutations in the genes encoding factor VIII (FVIII) (causing hemophilia A) or factor IX (FIX) (causing hemophilia B). The disease is characterized by recurrent bleeding, primarily into the joints and soft tissues. Currently, hemophilia is managed by intravenous infusion of clotting factor concentrates, which can be given either prophylactically or in response to a bleeding episode.

Liver-directed gene therapy for hemophilia has generally coalesced around the use of AAV vectors targeting hepatocytes, which can be used as protein factories to secrete the transgene

product into the circulation. The first liver-directed AAV trial for hemophilia B utilized a single-stranded AAV2 vector expressing human FIX infused via the hepatic artery into seven subjects (7). Efficacy was initially observed in the first of the two subjects that received the highest vector dose, 2×10^{12} vector genomes (vg)/kg, with peak FIX levels reaching ~10% of normal. Unexpectedly, an asymptomatic, self-limited rise in hepatic transaminases was observed beginning around week 4 after vector infusion; this coincided with the onset of a gradual loss of FIX activity. Both events were eventually attributed to the destruction of transduced hepatocytes by AAV capsid-specific memory $CD8^+$ T cells (59). None of the preclinical studies predicted the observed immune response to the capsid, and even after a decade of intense work the phenomenon remains imperfectly understood and not well modeled in animals (60). The other subject in the high-dose cohort yielded the second valuable lesson learned from that trial: Anti-AAV neutralizing antibodies, even at modest titers, prevent successful liver transduction when vector is delivered through the circulation.

Critical progress was achieved in the second liver-targeted AAV trial for the treatment of hemophilia B, which utilized a self-complementary vector genome packaged into an AAV8 capsid, administered by peripheral vein infusion (61, 62). All patients achieved long-term, stable FIX expression with average FIX levels of ~5% of normal in the patients in the high-dose cohort (2×10^{12} vg/kg), despite a transient increase in transaminases observed in 4 of 6 participants, likely as a result of a T cell response against the capsid. Notably, a short course of prednisolone reduced this response, and serum alanine aminotransferase levels returned to normal within days. The fact that this response was not observed at the two lower vector doses tested in this trial suggests that the immune response was dose dependent.

While the clinical improvement in patients who achieved stable FIX levels of ~5% of normal is indisputable, risk for excessive hemorrhage still exists. A recent study showed sustained vector-derived FIX:C in 10 participants all infused at the same dose, with mean steady-state FIX levels of 33.7% (63). This study utilized a single-stranded AAV vector consisting of a bioengineered capsid and a FIX-Padua (FIX-R338L) transgene, a naturally occurring FIX variant with increased specific activity, which allowed the use of a low vector dose (5×10^{11} vg/kg). On cumulative follow-up of 492 weeks, annualized bleeding rate and factor use were significantly reduced, as one would expect for patients with these factor activity levels. A total of 8 of 10 participants did not use factor, and 9 of 10 did not have bleeds after vector administration.

The development of AAV-based therapies for the treatment of hemophilia A is still at an early stage. One of the main challenges has been fitting the FVIII expression cassette within the restricted packaging limits of AAV, as the ~7-kb cDNA for FVIII exceeds the packaging capacity of AAV (~5 kb) (64). The use of a B-domain deleted form of FVIII (4.37 kb in size) circumvents this limitation but imposes a stringent constraint on the size of the regulatory elements that control FVIII expression. Importantly, therapeutic levels of circulating FVIII have been achieved in two recent phase I/II dose-escalation trials (NCT02576795, NCT03003533), accompanied by successful reduction in annualized bleeding rates after vector infusion with no reports of serious adverse events and no factor VIII inhibitors (65, 66). Trials for both hemophilia B and A have now entered phase III.

Recent clinical trials have established the promise of maintaining endogenous prophylaxis after a single gene therapy intervention, capable of near elimination of or significant reduction in factor usage and bleeding episodes, for hemophilia A and B. Extension of these results to patients with liver disease, preexisting anti-AAV neutralizing antibodies, or FVIII and FIX inhibitors—as well as the pediatric population, in which liver growth may reduce AAV-derived expression due to dilution of episomally maintained vector genomes—are longer-term goals for investigation. The success in liver-directed gene therapy for the hemophilias opens the door to potentially treating several inherited metabolic diseases and other plasma protein deficiencies. Studies are under way for

ornithine transcarbamylase deficiency (NCT02991144), homozygous familial hypercholesterolemia (NCT02651675), and mucopolysaccharidosis type VI (NCT03173521).

Gene Therapy for Central Nervous System and Neuromuscular Disorders

Despite preclinical data showing the feasibility of AAV-based gene therapy to treat CNS disorders, the number of clinical trials with encouraging results is relatively small. This is in part due to the anatomical and functional complexity of the human brain, including the existence of the blood–brain barrier, which limits biodistribution of the vector into the CNS. Even assuming successful delivery of the vector (e.g., via direct intraparenchymal, intrathecal, intracerebroventricular, or systemic administration), it is challenging to target a sufficient number of cells, either within a particular region or globally in the CNS, to achieve an adequate level of gene augmentation or gene suppression within a safe and therapeutic window.

The first clinical studies utilizing AAV vectors to mediate gene delivery to the human CNS were for the treatment of Canavan disease (67), Parkinson's disease (68), and late infantile neuronal ceroid lipofuscinosis (69). All these trials utilized AAV2 vectors delivered locally to certain areas of the brain; expression was confined to areas close to the injection sites, a desirable goal for Parkinson's but not for the other indications. Novel serotypes and different infusion techniques have been tested to improve vector distribution for those CNS indications that require global distribution of vector. Delivering AAV vectors into the cerebrospinal fluid, via either intracerebroventricular or intrathecal administration, has been proposed as a more efficient way to achieve global distribution (70).

A breakthrough study by Kaspar and colleagues demonstrated successful targeting of spinal motor neurons after systemic administration of AAV9 in neonatal mice (71). Using the same systemic intravenous administration of AAV9, these investigators subsequently showed robust efficacy in a phase I trial for the treatment of spinal muscular atrophy type 1 (SMA1) in 15 patients (72). SMA1 is the most common genetic cause of death during infancy, with less than 20% of patients alive and free of ventilatory support at 20 months of age (73). The 12 patients in the high-dose cohort demonstrated improvements in motor function and survival that were significantly different from the expected course of the disease, using natural history as a comparator. In particular, 11 have CHOP-INTEND scores higher than 40 points (Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders, an assessment designed to quantify the motor skills of infants with neuromuscular disorders), 11 have head control, 8 sit unassisted, and 2 can crawl, stand, and walk independently. Overall, this represents an impact on motor milestones seldom seen in this population (73). A phase III study is now ongoing (NCT03306277).

Some challenges in the development of successful CNS-directed therapies include the need for careful monitoring of potential immune responses, especially against the transgene product, and improved vector tropism in order to target higher numbers of cells at lower doses, as well as a more precise targeting of specific cell types in different regions of the brain. The landmark study in SMA1 patients, along with the favorable safety profile of CNS-targeted clinical trials in more than a decade of clinical experience using AAV vectors, warrants further development of this technology to treat complex multifactorial neurologic indications such as Parkinson's and Alzheimer's.

Genome Editing

This review has focused on augmentation gene therapy, in which a functional gene is transferred to a target tissue to drive expression of a gene product with therapeutic effects (**Figure 2**). Another compelling technique under development is genome editing, in which a mutation is corrected in situ, generating a wild-type copy under the control of the endogenous regulatory signals in

a physiologically relevant target tissue. This approach makes use of novel reagents including zinc finger nucleases, transcription activator–like effector nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (reviewed in 74). These are different types of sequence-specific nucleases that introduce double-stranded breaks into the DNA near the site of the mutation and then rely on a donated repair sequence and cellular mechanisms of DNA repair (e.g., homology-directed repair) to reconstitute or correct a functioning gene. Genome editing can also be used to knock down gene expression by introducing frameshift mutations at the site of DNA cleavage. However, only three in vivo gene therapy clinical studies are using a nuclease—for the treatment of hemophilia B (NCT02695160), mucopolysaccharidosis type I (NCT02702115), and mucopolysaccharidosis type II (NCT03041324)—and these do not aim to correct a mutation but rather take advantage of targeted integration of AAV donor cassettes into sites of DNA double-strand break at the albumin locus. The rationale behind this choice is to hijack the high transcriptional activity of the albumin promoter and use it as a safe harbor for integration (75). Since in adults the amount of hepatocyte turnover is relatively low and vector integration does not appear to be necessary to achieve durable expression levels after gene transfer (62), this approach may be better suited for treatment of pediatric patients. However, the two main ex vivo applications of genome editing aim at disrupting endogenous genes rather than integrating donor sequences. In particular, zinc finger nucleases (76) and CRISPRs (NCT03164135) have been used to disrupt the HIV-1 coreceptor CCR5 to treat HIV infection. CRISPRs also gaining traction to modify T cells for the immunotherapy of cancer (NCT02793856).

CONCLUSION

The power and versatility of gene transfer strategies are such that there are few serious disease entities for which gene transfer therapies are not under development. The development of new classes of therapeutics typically takes two to three decades; monoclonal antibodies and recombinant proteins are recent examples. Gene therapeutics, which entered clinical testing in the early 1990s, traversed the same time course. Examples of clinical success are now ample, and gene therapy approaches are likely to become increasingly important. A central question is the long-term safety of gene transfer, and regulatory agencies have mandated a 15-year follow-up for subjects enrolled in many gene therapy trials. Realization of the therapeutic benefits of modern molecular medicine will depend on continued progress in gene transfer technology.

DISCLOSURE STATEMENT

Dr. Anguela and Dr. High are employees of, hold equity in and are coinventors on patents licensed to Spark Therapeutics, Inc.

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