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Gene Therapy 2017: Progress and Future Directions

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REVIEW

Gene Therapy 2017: Progress and Future Directions

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INTRODUCTION: GENE THERAPY IN 2017

Gene therapy has changed dramatically in the 28 years since the first human gene transfer experiment in 1989. Alipogene tiparvovec, GlyberaR®, a recombinant adeno-associated virus (rAAV) product for lipoprotein lipase deficiency, and Strimvelis®, a lentivirus vector for severe combined immune deficiency are approved in Europe. An rAAV2 product for a congenital form of blindness is currently under review in the United States, likely to be followed by numerous other gene therapies.

Nonviral gene transfer

The success of gene therapy has largely been driven by improvements in nonviral and viral gene transfer vectors. An array of physical and chemical nonviral methods have been used to transfer DNA and mRNA to mammalian cells and a substantial number of these have been developed as clinical stage technologies for gene therapy, both *ex vivo* and *in vivo*.

Cationic liposome technology is based on the ability of amphipathic lipids, possessing a positively charged head group and a hydrophobic lipid tail, to bind to negatively charged DNA or RNA and form particles that generally enter cells by endocytosis. Some cationic liposomes also contain a neutral co-lipid, thought to enhance liposome uptake by mammalian cells.[4–7] Similarly, other polycations, such as poly-l-lysine and polyethylene-imine, complex with nucleic acids via charge interaction and aid in the condensation of DNA or RNA into nanoparticles, which are then substrates for endosome-mediated uptake.[8] Several of these cationic-nucleic acid complex technologies have been developed as potential clinical products, including complexes with plasmid DNA (pDNA), oligodeoxynucleotides, and various forms of synthetic RNA.[9–11]

Modified (and unmodified or "naked") DNA, RNA, and oligonucleotides have also been shown to mediate successful gene transfer in a number of circumstances. These include the use of pDNA by direct intramuscular injection for DNA vaccines, the use of intratumoral injection of pDNA to deliver cytokine and/or suicide genes, systemic (s.c. or i.v.) injection of antisense nucleotides to induce RNAse H1 or exon-skipping.[12–14] The most recent of these developed for induction of RNAi are discussed in a later section.

Ex vivo introduction of pDNA and/or other nucleotides using physical methods has been well developed for certain cell types, including T lymphocytes.[15] Electroporation techniques have become the standard with T cells for the introduction of a variety of molecular cargoes, including ribonucleoproteins composed of Cas9 and short-guide RNAs for genome editing (see section below) and transposons for long-term integration of transgenes.

Gammaretrovirus and lentivirus vectors

Many within the gene therapy field consider viruses as the ultimate vectors for the delivery of therapeutic tools, and the number of gene therapy clinical trials reflects this bias.[16–20] Retroviruses were the first class of viruses to be harnessed for mammalian and human gene transfer, and they are at the leading edge of products that show clinical efficacy[1] (**Figure 1**).

For example, direct clinical benefit with chimeric antigen receptor T (CAR-T) cells is a promising novel therapy for many malignancies. CAR-T cells are produced by *ex vivo* transduction of T cells with lentiviral vectors.[21–23] Exciting results with B-cell lymphomas and leukemia eradication was seen when CAR-T cells are directed against the B-cell surface antigen, CD19.[24,25] However, because CD19 is a pan-B cell marker, one side effect is normal B-cell depletion. Thus, to try and restrict normal B-cell depletion after CAR-T cell administration, a recent study refined CD19 CAR-T cells to recognize κ-restricted cells, thereby excluding normal B-cells from targeted destruction.[26] In addition, other tumor-associated antigens have been targeted with some clinical success.[21,27,28] Although most of these trials have utilized autologous T cells, one recent report showed efficacy in "off-the-shelf" (TCR$^-$/CD52$^-$) allogeneic anti-CD19 CAR-T cells. These T-cells not only are transduced with the lentivirus expressing a chimeric antigen receptor, but they also have their endogenous T-cell receptor knockout via transcription activator-like effector nuclease TALEN-mediated genome editing.[29,30]

Another prominent example of clinically effective gene therapy with gammaretrovirus and lentivirus vectors is *ex vivo* transduction of hematopoietic stem cells to treat conditions such as severe combined immunodeficiency (SCID). These include both X-linked SCID gammaretrovirus[31] and lentivirus[32] therapies, as well as SCID due to adenosine deaminase-SCID deficiency. In fact, the lentiviral Stimvelis^R recently received European Market Authorization to treat patients with adenosine deaminase-SCID deficiency.[33] In

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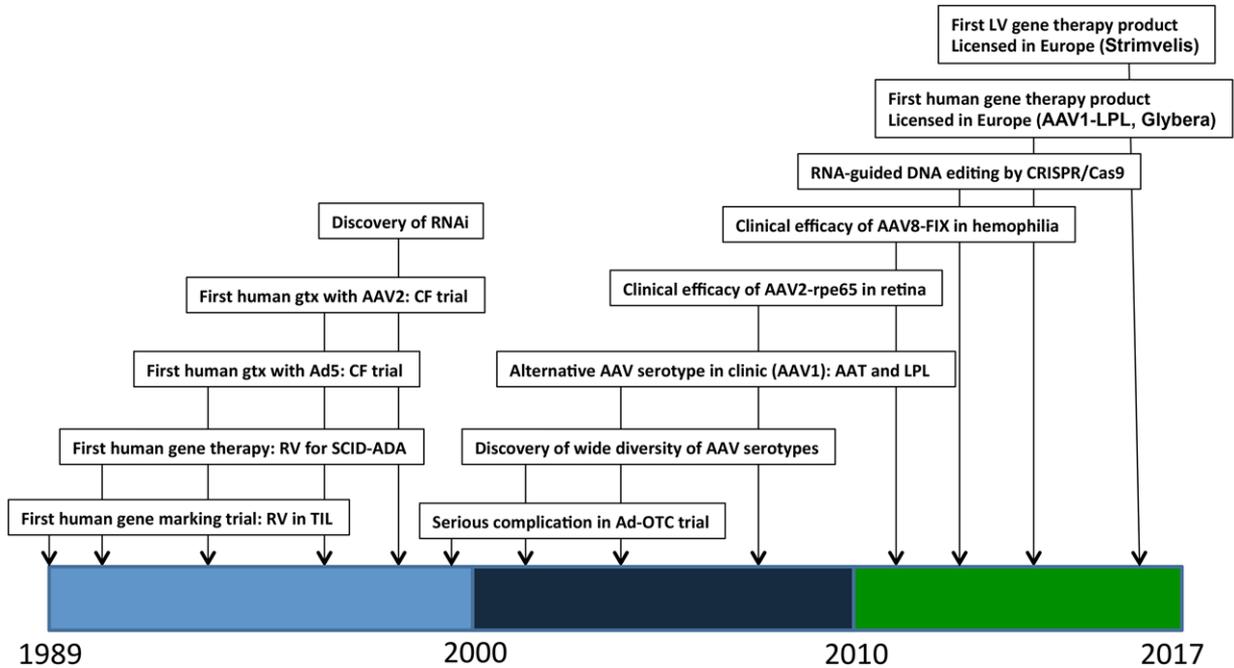
Figure 1 Timeline of major events in clinical gene therapy. A few selected key dates in the history of human gene therapy are depicted, with the dates indicated on the x-axis. AAV2, adeno-associated virus type 2; Ad5, adenovirus type 5; CF, cystic fibrosis; CRISPR, clustered regularly interspaced short palindromic repeats; FIX, clotting factor IX; LPL, lipoprotein lipase; OTC, ornithine transcambamylase; RNAi, RNA inhibition; RPE65, 65 kilo-Dalton retinal pigment epithelial protein; RV, gammaretrovirus; SCID-ADA, severe combined immune deficiency due to adenosine deaminase deficiency; TIL, tumor-infiltrating lymphocytes.

Table 1 Gene therapy clinical trials for monogenic diseases in phases III/IV

Disease	Vector	Outcomes	Location of the Trial	References
LPLD	AAV-Glybera	Improved lipid profile and decreased pancreatitis	The United States	113–115
X-linked adrenoleukodystrophy	Lentivirus	Improved neurologic development	France	19
Thalassemia major	Lentivirus	Decreased transfusion need	The United States	16
X-linked/ choroideremia rentinal disease (REP1)	AAV2	Improved vision	The United States- multiple countries	17,116
LCA	AAV2	Improved low-light vision	The United States	20,117,118
Leber hereditary optic neuropathy	AAV2	Improved vision	France - multiple countries including US	18,119

AAV, adeno-associated virus; LCA, Leber congenital amaurosis type 2; LPLD, lipoprotein lipase deficiency.

addition, similar clinical effectiveness was seen in X-linked adrenoleukodystrophy between patients treated with *ex vivo* lentiviral correction and those treated with allogeneic hematopoietic cell transplantation (**Table 1**).[19] Other promising retroviral hemopoietic stem-cell gene therapies include lentiviral therapies for metachromatic leukodystrophy[34] and both gammaretroviral and lentiviral therapies for Wiskott-Aldrich syndrome. In Wiskott-Aldrich syndrome, lentiviral therapies showed a safer profile than gammaretrovirus vectors, relative to the risk of insertional mutagenesis.[35,36]

Adenoviruses and oncolytic viruses

Adenoviruses (Ads) were also used early on in gene therapy clinical trials, and are one of the most studied and published viral vectors (**Figure 1**). Ads have robust transduction profiles, particularly in the liver, but they were also accompa-

nied by robust immune responses. Different levels of attenuation of the virus can be achieved by removing different components, including complete removal of all genetic information – the so-called "gutless" vectors.[37] Unfortunately, early clinical trials for gene correction using Ads did not have many clinical successes, and one trial resulted in a tragic fatality.[38] Additional hurdles seen with systemic delivery include nonspecific binding to blood components leading to viral inactivation. In addition, a majority of adults have antibodies against common Ad5 serotypes.[39,40] Further modifications of Ad vectors, such as making chimeric vectors, and chemical modifications have helped overcome some of the early challenges with liver targeting and host immunity.[37] However, Ads have recently been used in cancer treatment as oncolytic viruses. A number of clinical trials using Ad to target a number of different cancers, such as

prostate, ovarian, bladder, and refractory solid tumors, have been promising.[41–45] In this type of therapy, robust immune responses are beneficial for therapeutic outcomes. Many other viruses have been used as oncolytic viruses, such as: vaccine virus; herpes virus; Coxsackievirus, reovirus, parvovirus, vesicular stomatitis virus, Newcastle disease, measles virus, polio virus, and Seneca Valley virus.[46] The first clinically approved oncolytic virus is Talimogene Laherparepvec (Imlygic®; Amgen, South San Francisco, CA), which is a genetically modified herpes virus expressing human granulocyte-macrophage colony-stimulating factor. Talimogene Laherparepvec has been approved for the treatment of advanced melanoma.[47–50] Thus, Ad, one of the earliest vectors in the gene therapy field, may find a new role in cancer gene therapy along with other oncolytic viruses.

Recombinant adeno-associated virus vectors

Recombinant adeno-associated virus (rAAV) vector-mediated gene therapy has also proven to be efficacious in certain conditions.[2] Hundreds of clinical trials have been performed using rAAV viral vectors for recessive monogenic disorders, with the first human rAAV injection performed almost 25 years ago.[51] Examples of clinical efficacy with rAAV include data from trials with hemophilia B,[52,53] spinal muscular atrophy (unpublished), alpha 1 antitrypsin,[54,55] and Leber congenital amaurosis.[56] In hemophilia B, a single systemic administration of rAAV carrying the human factor IX gene resulted in a multiyear sustained expression of factor IX levels at 1–6% of normal.[52,53] Similarly, in alpha 1 antitrypsin, intramuscular injections of AAV1 carrying the *AAT* gene resulted in sustained AAT expression for 5 years (Gruntman *et al.*, unpublished). Ongoing clinical trial in spinal muscular atrophy resulted in improved survival in patients with spinal muscular atrophy type 1. Patients with Leber congenital amaurosis had partial restoration of their vision after receiving therapy with rAAV2 vector carrying the human retinal pigment epithelium 65kDa gene.[55]

Other rAAV vectors are on their way through preclinical and clinical proof-of-concept studies **Table 1**. Recently, a number of proof-of-concept studies have been completed using rAAV technology for correction of single gene disorders targeting a wide variety of tissues. The clinical success of the Leber congenital amaurosis trial, have led to a number of studies targeting genetic diseases of the retina.[3] Diseases of the central nervous system have also had some important proof-of-concept studies, along with metabolic and skeletal diseases.[57–62] Additionally, rAAVs have been used to treat diseases other than monogenic disorders, such as interferon-beta delivery to treat the aggressive brain cancer glioblastoma multiforme,[63] and to provide treatment for infectious diseases, such as human immunodeficiency virus[64] and influenza.[65]

Despite these many successes with rAAV and other viral gene therapies, the future of the field of gene therapy may lie in new technologies. Examples of these technologies stem from the discovery of RNA interference in *C. elegans* by Fire *et al.*,[66] discovery of the host defense system CRISPR/Cas9 in *S. pyogenes*,[67] and finally from the discovery of new vectors through co-evolution and directed evolution.
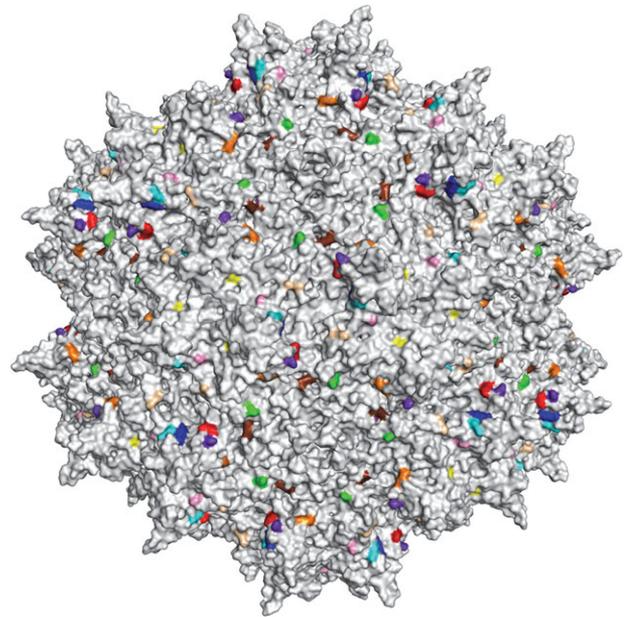

Figure 2 Structure of adeno-associated virus 2 (AAV2).[113] The icosahedral structure of the virus capsid is shown. Note that this structure is symmetrical across a twofold, threefold, and fivefold axis of symmetry. The ability of the AAV vectors to transduce various cell types largely depends on variation in amino acid moieties highlighted in color.

Novel adeno-associated virus capsids

The rAAV is a simple and ubiquitous wildtype virus that occurs naturally in humans (**Figure 2**). Even wildtype adeno-associated virus (AAV) is naturally a vector given its replication dependence on helper viruses. However, antibodies against AAV can decrease the efficacy of rAAV-mediated gene therapy. A lot of work has recently gone into updating the existing repertoire of natural variants of AAV viruses by both rational design and directed evolution. For example, pioneering work by Gao *et al.*,[68,69] greatly added hundreds of natural variants to the gene therapists tool kits and new AAV variants are still being discovered. In addition, many novel vectors have been rationally engineered. These new vectors allow avoidance of the immune system and enable tissue-specific tropism to target exact organs involved in the disease of interest. Rationally engineered viruses, such as AAV2g9, have been developed to exploit the benefits from parental variants, such as the galactose receptor footprint from AAV9, while creating unique transduction profiles, such as central nervous system-restricted transduction.[70,71] Furthermore, directed evolution was used to create unique transduction profiles. Using a cre-recombination-based adeno-associated virus-targeted evolution (CREATE) strategy, novel AAV variants can now achieve widespread expression through the central nervous system in mice.[72] However, improved transduction efficiency is not only limited to the CNS but has also been more efficient in muscles,[73] as well as in human and murine livers.[74]

RNAi

The discovery of RNAi allowed for a shift from gene therapy focused on gene augmentation to a focus on downregula-

tion of gene expression for diseases in which pathology is caused by toxic gain of function. Some of the first clinical therapies used small-interfering RNA in diseases of the liver; small-interfering RNA sequences were developed to target hepatocytes[75] in which knockdown of gene expression have a therapeutic effect on disease pathogenesis. Examples of this include therapy for transthyretin-mediated amyloidosis[76] and complement-mediated diseases.[77]

However, in order to achieve long-term knockdown, a more stable approach is to use another RNAi pathway, specifically delivery of synthetic microRNA (miRNA), which can be continuously expressed by viral vectors. Interestingly, the discovery of exogenous RNA-mediated downregulation by Fire *et al.*,[66] was preceded by the discovery of miRNA in *C. elegans* by Lee *et al.*[78] and Wightman *et al.*[79] Both discoveries utilize the same enzymatic pathway that allows for exploitation of natural miRNA in mammalian systems for therapeutic purposes. The rAAV delivery platform can be used to continually express synthetic miRNA "genes" to downregulate a gene by sequence-specific targeting. This technology has been used to treat dominant disorders, such as Huntington's disease, in which a toxic gain of function causes neurological disease by increased number of CAG repeats in the huntingtin's gene.[80–82] It has also been used to treat SOD1-mediated amyotrophic lateral sclerosis in a murine model of amyotrophic lateral sclerosis, in which motor neuron disease is caused by toxic gain of function of the SOD1 protein.[58,83] Furthermore, this system can be used to treat diseases in which both a loss-of-function and a toxic gain of function is associated with a certain mutation. For example, in alpha-1 antitrypsin (AAT) deficiency, a mutant AAT protein (the PiZ protein) causes both liver disease by toxic accumulation of the protein and emphysematous lung disease by an absence of AAT. For AAT, rAAV was used to deliver a miRNA designed to knock-down expression of PiZ causing liver disease while simultaneously augmenting expression of the normal AAT transgene to prevent lung disease.[84]

CRISPR/Cas9 genome editing

Novel exciting gene editing tools offer a more elegant and precise method of treating genetic diseases. There have been efforts on this front through *ex vivo* homologous recombination, TALENS and Zinc Finger Nucleases. However, none have the promise of the recently discovered clustered regularly interspersed short palindromic repeats (CRISPR)/crispr-associated protein 9 (Cas9).[67] CRISPR/Cas9 has transformed biomedical research. This technology was initially discovered in bacteria and archaea as a means for these organisms to defend themselves against invading viruses.[85] Using CRISPR/Cas9, many are targeting specific regions of the human genome in an attempt to achieve a therapeutic effect.[86–88] Targeting sequence specificity, similar to that of the RNAi approach, allows for the success and efficiency of CRISPR/Cas9.[89–91] In order for the system to work, synthetic short-guide RNAs are delivered in combination with a Cas-like enzyme, which allows for double-stranded breaks in the host DNA in a specific manner. Expression of genes can be disrupted by host DNA repair mechanisms in which a small insertion and/or deletion (indel) of two to six nucleotides generally results in a frameshift mutation and termination.[86,87]

However, genes can also be repaired by providing a ds-DNA template with the short-guide RNA and Cas-like enzyme, allowing homology-dependent recombination to occur.

Although CRISPR/Cas9 therapies are still in early development, some therapeutic approaches have already been demonstrated for genetic diseases and for Duchenne muscular dystrophy[92] and liver disease fumarylacetoacetate hydrolase deficiency.[93] One of the major challenges is in the delivery of the components necessary to complete the editing process. Viral vectors like rAAV have been suggested, but long-term expression, one advantage of rAAV, in augmentation or downregulation therapy becomes a disadvantage in the CRISPR/Cas9 genome editing in which short-term expression is all that is necessary to make the genetic changes. Conversely, vectors like adenovirus, which have robust expression albeit only in the short term, would be ideal if immune responses were not robust and led to clearance of virally targeted cells. However, the remaining challenges should not dissuade scientists from perusing these types of therapies as the potential for several clinical applications are impressive.

CONCLUSION/FUTURE DIRECTIONS

The knowledge gained within the field over the past several decades provides much hope for the future of gene therapy. The exciting possibility of treating many genetic and infectious disorders is now close to a reality with the success of rAAV in bench-work and clinical trials, novel vector engineering, and the recent discoveries of miRNAs, and CRISPR/Cas9. We are on the brink of having therapies approved for clinical usage in the United States, and the European Medicines Agency already has two approved therapies. AAV encoded miRNAs will soon be tested in clinical trials, whereas technologies, such as CRISPR/Cas9, are still in proof-of-concept stages but hold massive clinical promise.

Moving forward, the ability to exploit new molecular tools, such as RNAi and CRISPR/Cas9, should be able to make use of some of the clinical development paradigms from earlier gene therapy trials. Examples of this might include basic preclinical and clinical study designs to examine biodistribution of vector components and risk for carcinogenesis. One important distinction exists, however, between conventional viral gene therapy vectors and newer RNA-guided mechanisms. The toxicity observed with viral vectors has generally been consistent with toxicity of the viruses on which each vector is based.[94] This is true of both Ad vector-mediated inflammation and gammaretrovirus vector-mediated leukemia.[95–97] In the case of CRISPR/Cas9, a system is being used that does not occur in mammalian cells at all, as far as is currently known.[67,98] Likewise, although miRNA-mediated gene regulation is seen in mammalian cells, the understanding of miR-based diseases is at a fairly early stage. Thus, the modelling possible toxicities from such therapies are based more on theoretical concerns than on past experiences.

Nonetheless, it seems likely that certain genetic diseases will be approachable with CRISPR/Cas9 and RNAi-based therapy that were not approachable with prior methods.[86–88,92] Specifically, the ability to treat autosomal

dominant disorders is a feature of both of these methods. In addition, CRISPR/Cas9 presents the ability to repair a gene *in situ*, allowing for preservation of all of the elements required for normal physiologic regulation of the gene of interest by its own promoter and enhancers.[99,100]

This has led some to speculate that CRISPR/Cas9 could actually enable a permanent and definitive germ-line correction of a genetic disorder.[101,102] One such study, performed in nonviable human embryos, demonstrated the feasibility of doing so in addressing hemoglobinopathies.[103] Clearly, such an approach is not currently deemed to fall within ethical guidelines,[102] although some have pointed out that therapeutic transfer of whole mitochondria has been allowed even though mitochondrial DNA will likely be passed down in the germ line. It is conceivable, however, that a purely therapeutic approach, intended to cure a disease rather than to enhance, could be allowable in the future if appropriate questions about safety and efficacy can be addressed. This was anticipated by the joint statement from the National Academy of Sciences and the National Academy of Medicine.[104] If that were to transpire in the future, it could represent the most definitive treatment for families with a genetic defect that has ever been attempted.

More near-term, the ability to control gene expression in the context of gene transfer may be a more realistic goal. A number of promoter systems inducible by small molecule drugs have shown excellent dynamic range for gene regulation in cell culture and in animal models. Among these are the tetracycline inducible and repressible systems, and those based on modified estrogen and progesterone receptors.[105–108] None of these has achieved clinical translation to date, primarily due to the immune responses to transcriptional activator domains that are not human in origin.[109–111] The field of synthetic biology has produced two-component RNA-based systems, so-called "toehold switches" that may have superior characteristics in certain circumstances.[112] The added complexity of these multicomponent systems could be particularly useful for transgene products with a relatively narrow therapeutic window of expression. This sort of adaptation of different gene therapy platforms to different disease targets will likely represent the primary challenge and opportunity facing gene therapy researchers in the coming years.

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