# From unique challenges to inspired solutions: how vector engineering makes *in vivo* gene therapy possible

**Rodanthi Lyraki**

Recent advances in designing safe and efficient viral vectors have spearheaded unprecedented growth in clinical trials and regulatory approvals for experimental gene therapies. Still, important challenges remain for gene therapies that require systemic administration into the circulation, including the patient's immune response and the need for precise activation in the target cell population while sparing other tissues. Extensive research in engineering the vector's external surface and genome has led to clever solutions, allowing novel *in vivo* therapies for previously untreatable genetic diseases such as spinal muscular atrophy and Duchenne muscular dystrophy to reach the clinic. In the future, we expect novel strategies, such as different viral vectors or non-viral delivery vehicles, to overcome the shortcomings of viral therapy.

Even though the concept of gene therapy has fascinated scientists ever since the 1970s, it is only in the last decade that a real explosion of clinical trials and regulatory approvals has taken place, mainly thanks to recent achievements that have made it possible to minimize safety concerns. Gene therapy is defined as the transfer of genetic material into cells with the aim of replacing, modifying or changing the expression of a faulty gene. While genetic diseases linked to a single mutated gene are uniquely suited targets of gene therapy, it has been most successfully applied to cancer immunotherapy. Recently approved drugs such as Yescarta and Kymriah rely on the modification of patients' cells in the lab and subsequent transplantation back into the body. However, most genetic diseases require administering therapeutic agents directly inside the patient's body, targeting the liver, heart, muscle or other organs (*in vivo* therapy). This type of gene therapy presents unique challenges requiring ingenious solutions that led to the development of a new field of applied molecular biology: vector engineering.

Currently, most applications employ viral vectors as the delivery method of choice. This way, we take advantage of the virus's natural ability to transfer its genetic material into target cells (transduce) and hijack the cell's machinery to produce proteins required for its own propagation. Decades of biosafety research and extensive genome modification have turned viruses into viral vectors: these are harmless vehicles for delivering therapeutic genes without substantial risk of multiplication or infection. In fact, the majority of the viral genome in these vectors is replaced by an expression cassette, which typically includes minimal viral DNA sequences (required for integration or assembly within host cells) and the therapeutic gene of choice bordered by regulatory sequences that dictate its expression profile: in which tissues the desired protein will be actively produced and how abundantly. Examples of regulatory sequences are the combination of promoter and enhancer and a transcription termination signal such as a poly(A) tail (Figure 1).

## Designing an efficient *in vivo* gene therapy

Researchers must choose an appropriate route of administration for viral vectors to reach the target organs, such as intravenous infusion. In this case, one of the first hurdles we meet is the body's natural inclination to mount a robust immune response against the intruder elements. The immune system can recognize both the external surface of the virus and the therapeutic transgene as foreign. Not only does this response cause toxicity, but it can also undermine any therapeutic effort by eliminating the viruses or the transduced cells.



 

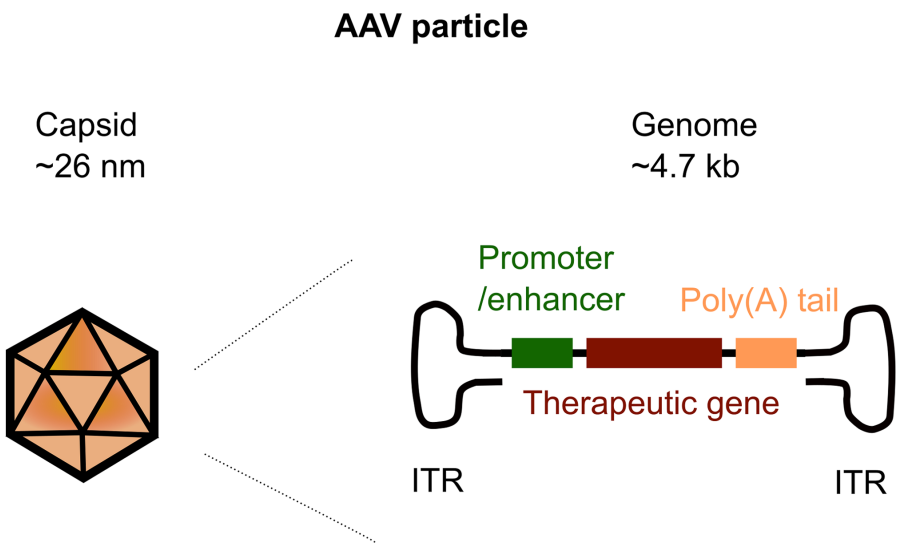


**Figure 1.** The expression cassette of an AAV vector containing viral sequences (inverted terminal repeats, ITR), regulatory sequences and a therapeutic coding sequence.

A second goal when designing an *in vivo* gene therapy is ensuring the therapeutic transgene's expression is fine-tuned to the correct cell population while sparing off-target tissues. This specificity depends on the vector's capacity to transduce target cells with high efficiency (tropism) and regulatory elements such as the promoter. Ideally, the expression should be long-lasting and endure cycles of proliferation and organ growth because the extremely high cost of gene therapies and the potential of immune memory might prohibit repeat administrations. Moreover, integrative viruses pose a risk of insertional mutagenesis such that neighbouring genes can be disrupted or have their expression aberrantly driven by strong viral promoters. Finally, not all therapeutic sequences can be packaged inside a viral vector (e.g., some might be too large).

## Spinal muscular atrophy: systemic delivery to achieve broad expression

Treatment of the severe paediatric disease spinal muscular atrophy (SMA) is one of the most hopeful applications of *in vivo* gene therapy. SMA is a genetic disease. Mutations in the gene *SMN1* (survival motor neuron 1) impact protein SMN's function leading to the degeneration of specialized nerve cells called motor neurons. Since motor neurons in the brain and spinal cord control muscle movement, their loss causes irreversible muscle weakness. Infants with SMA fail to reach developmental milestones and to perform basic functions like swallowing and breathing.

Earlier attempts at gene therapy to combat the disease involved direct administration to the central nervous system of small DNA molecules called antisense oligonucleotides (medicine marketed as Spinraza). Instead of replacing the faulty gene, oligonucleotides modify the expression pattern of a similar gene called *SMN2* with the aim of using it as a template to produce increased levels of SMN protein. Although this represented an innovative solution, it was limited by its narrow tissue distribution. SMN protein is found not only in the nervous system but also in various other tissues, including the heart, pancreas and skeletal muscles. To combat these limitations, scientists took advantage of the broad tropism of adeno-associated viruses (AAVs). AAVs are small viruses whose single-stranded DNA genome is surrounded by a protein structure called a capsid. They are the vehicle of choice for many *in vivo* gene therapy studies because of their safety profile, as they are naturally non-pathogenic and do not integrate into the genome but remain in the nucleus as circular DNA entities called episomes.

Notably, one particular AAV subtype (serotype), the AAV9, can not only transduce tissues such as the lung, heart and muscles efficiently, but it can also cross the blood–brain barrier and deliver the therapeutic product to the central nervous system. AAV9 in the medicine marketed as Zolgensma (approved in 2019 and 2020 in the USA, Japan and Europe) carries a healthy version of the *SMN1* gene and regulatory sequences that drive its expression as broadly as possible (the chicken β-actin promoter). In addition, instead of a single-stranded genome that needs to be converted to double-stranded before transduction, the virus in Zolgensma comes in a self-complementary form which can be transcribed immediately. This clever modification increases transduction efficiency. Thanks to these interventions, treated infants survived longer

and achieved developmental milestones impossible for untreated patients, such as sitting, feeding and walking. Although these are significant improvements to patients' lives, Zolgensma is not a universal cure, but its efficiency increases the younger the patient at the time of administration. As with all gene therapies, the astronomically high price (about $2m per dose) remains an important issue.

## Duchenne muscular dystrophy: the challenge of delivering a large gene to the entire muscular system

Duchenne muscular dystrophy (DMD) is another rare genetic disease that causes debilitating muscle wasting during infancy. DMD is caused by mutations in the gene encoding for dystrophin, a protein found in skeletal, smooth and cardiac muscles where it is necessary for maintaining their structure. The simple premise of delivering a healthy version of the dystrophin gene to muscle cells using viral vectors stumbles on two crucial obstacles. First, dystrophin is encoded by the largest human gene (*DMD*); the dystrophin coding sequence is twice as large as the AAV genome. However, as early as 30 years ago, researchers discovered patients who suffered from only a mild form of dystrophy despite lacking a considerable part of dystrophin. Based on the extensive research that followed this initial observation, protein regions necessary for its function were isolated and assembled into a kind of 'mini gene' named microdystrophin. In June 2023, the FDA granted accelerated approval to an AAV-based approach (named Elevidys) to deliver human microdystrophin to patients 4–5 years of age based on evidence of expression in target tissues and with the expectation of future trials showing clinical benefit.

Second, since AAV naturally concentrates in the liver, delivering sufficient quantities to the skeletal muscle, which covers about 40% of the whole body, is challenging. To ensure microdystrophin is not expressed in the liver, researchers involved in the recently approved therapy used regulatory elements that drive expression specifically in the cardiac and skeletal muscles (called the MHCK7 promoter/enhancer) (Figure 2). Still, very high quantities of the virus must be administered to achieve sufficient muscle transduction, entailing a substantial liver toxicity risk. In its quest to improve transduction specificity and efficiency, the scientific

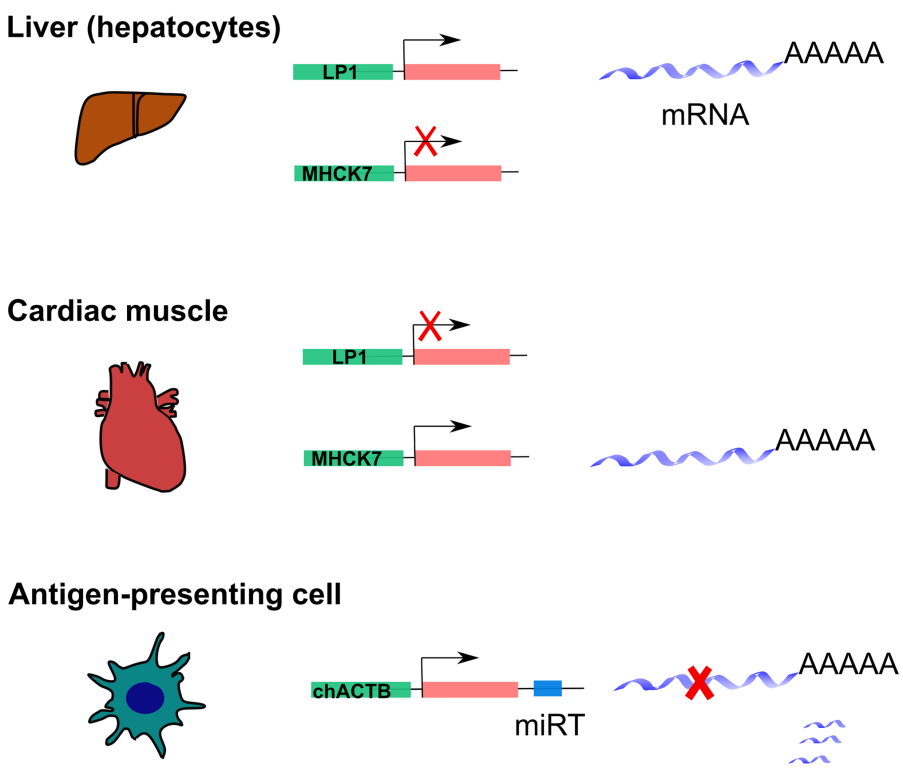


**Figure 2.** Achieving specific expression in gene therapy via the use of tissue-specific promoters and miRNA target sequences. An artificial promoter (LP1) activates the therapeutic coding sequence in hepatocytes and a few other tissues, while another artificial hybrid promoter (MHCK7) drives activity in skeletal and cardiac muscles. Adding a particular miRNA-142 target sequence (miRT) leads to the downregulation of expression in APCs, even when the expression is driven by a ubiquitous promoter (chicken β-actin).



 

field has engineered the external capsid to achieve better muscle tropism. In one study, a method of identifying novel artificial capsid sequences called 'directed evolution' created variants showing at least 10 times increased preference for muscle cells and minimized liver transduction. Such approaches will undoubtedly improve muscle-targeted *in vivo* gene therapies in the future.

## Bypassing host immune responses and the treatment of haemophilia

The patient's immune response is a significant barrier to the long-term sustained effectiveness of AAV therapies. This extensively studied response includes immediate, first-line immune responses and delayed, specialized responses against the capsid and the therapeutic protein. These delayed responses, known as 'adaptive', involve the production of antibodies. In fact, antibodies against the AAV capsid are quite common in the human population, as many of us have been exposed to this virus before. Even if patients do not have pre-existing antibodies, they will surely develop them after therapy, preventing re-administration. Thus, therapy must be one-off and for life. Several ways have been proposed to circumvent this problem, including using drugs that dampen the immune response, such as rapamycin, although this drug regulates a paramount metabolic pathway and is inevitably associated with potential side effects. A common strategy is selecting candidates for therapy who lack such serum antibodies. Moreover, antibody presence depends on the AAV serotype. For the DMD trial, scientists used an AAV serotype of primate origin which should reduce the chance of pre-existing immunity among patients.

Since many of the viral particles gather in the liver after intravenous administration, it is not surprising that hepatotoxicity is a common unwanted effect of AAV therapy. This secondary effect, usually successfully treated with corticosteroids, is traced back to another component of adaptive immunity, the cytotoxic T lymphocytes (CTLs), a class of blood cells which eliminate transduced cells. All types of transduced cells can act as accomplices, presenting pieces of foreign elements on their surfaces for the CTL to recognize. Still, one cell type is of special interest to gene therapy immunology: the professional antigen-presenting cells (APCs). Macrophages, dendritic cells and B cells can all act as APCs. In this case, vector engineering of the regulatory elements can help dampen the immune response.

Haemophilia B is a genetic disorder where a deficiency of protein coagulation factor IX (encoded by the *F9* gene) prevents normal blood clotting. Providing a normal copy of the *F9* gene to patients' hepatocytes by AAV-mediated gene therapy can significantly reduce bleeding episodes compared to traditional therapy (continuous replacement of external factor IX, an expensive and burdensome procedure). An ideal therapy designed to achieve maximal therapeutic expression and minimal immunogenicity would specifically target the liver while sparing APCs. To this end, various studies have used liver-specific promoters, natural or artificial, combining regulatory elements from different genes to achieve the desired expression levels. Further, a clever solution was devised after discovering a microRNA molecule uniquely expressed in progenitors of professional APCs. MicroRNAs (miR) are small non-coding RNAs that contribute to tissue specificity by repressing the expression of specific genes. Researchers can selectively exclude the therapeutic protein from APCs by incorporating an miR target sequence in the expression cassette (Figure 2). This promising approach, so far tested in animal models, is only one of many similar capsid and transgene engineering approaches expected to ameliorate immune response in future gene therapies.

## A look to the future

Although most *in vivo* attempts that report successful clinical trials have used AAV vectors, future advances are expected to enable the use of lentiviral vectors (LVs), already supported by successful *ex vivo* applications to treat blood diseases and substantial pre-clinical research. LVs are highly engineered vectors derived from the human immunodeficiency virus (HIV). Apart from their capacity to integrate into the genome, allowing for a stable expression of the therapeutic gene even in highly replicative organs, they showcase other potential advantages. These vectors show low immunogenicity (pre-existing immunity to HIV is low in human populations) and an increased capacity to accommodate exogenous sequences up to 8 kb. Several biosafety measures assure a satisfactory safety profile for LVs. For example, LVs are not disease-causing because a large part of the virus's genome, critical for producing infectious particles, has been deleted in vectors used for experimental therapies. Still, their use in clinical settings has been delayed by difficulties in producing sufficient amounts for *in vivo* use. Finally, many efforts are underway to escape the use of viruses altogether. These 'virus-free' gene therapy vehicles consist of lipid or protein nanoparticles or even circular DNA molecules. While the field is still in its infancy, there is hope that it can overcome the main shortcomings of viral vectors, including the high cost and prohibition of repeat administrations. ■

## Further reading


- Conroy, G. (2022) How gene therapy is emerging from its 'dark age.' *Nature*, **612**, S24–S26. doi: 10.1038/d41586-022-04210-5
- Tang, R., and Xu, Z. (2020) Gene therapy: a double-edged sword with great powers. *Mol. Cell. Biochem*. **474**, 73–81. doi: 10.1007/s11010-020-03834-3
- Annoni, A., Gregori, S., Naldini, L., and Cantore, A. (2019) Modulation of immune responses in lentiviral vector-mediated gene transfer. *Cell. Immunol*. **342**, 103802. doi: 10.1016/j.cellimm.2018.04.012
- Tabebordbar, M., Lagerborg, K.A., Stanton, A., et al. (2021) Directed evolution of a family of AAV capsid variants enabling potent muscle-directed gene delivery across species. *Cell*, **184**, 4919–4938.e22. doi: 10.1016/j.cell.2021.08.028
- Li, C., and Samulski, R.J. (2020) Engineering adeno-associated virus vectors for gene therapy. *Nat. Rev. Genet*. **21**, 255–272. doi: 10.1038/s41576-019-0205-4
- Reardon, S. (2023) "It's a vote for hope": first gene therapy for muscular dystrophy nears approval, but will it work? *Nature*, **618**, 451–453. doi: 10.1038/D41586-023-01799-Z
- Zufferey, R., Dull, T., Mandel, R.J., et al. (1998) Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J.Virol*. **72**, 9873–9880. doi: 10.1128/JVI.72.12.9873-9880.1998
- Sheridan, C. (2023) Why gene therapies must go virus-free. *Nat. Biotechnol*. **41**, 737–739. doi: 10.1038/S41587-023-01824-6
- National Hemophilia Foundation (2022) FDA approves first gene therapy to treat adults with Hemophilia B, https://www.hemophilia.org/news/first-hemophilia-b-gene-therapy-approved-by-fda


*Dr Rodanthi Lyraki is a molecular biologist and science writer, passionate about uncovering the foundations of human disease. She studied biology in Athens as an undergraduate, then completed a Master's in Biomedical Sciences at the University of Edinburgh and a PhD on the molecular mechanisms of inherited retinal degeneration. She obtained a postdoctoral fellowship from the Fondation pour la Recherche Médicale in France to study sexual dimorphism in adrenal cortex physiology and cancer. Her recent involvement with the vector engineering team of the French start-up Innoskel sparked her interest in gene therapy advances.*
*Email: rlyraki@gmail.com*



 