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Review

Emerging Issues in AAV-Mediated *In Vivo* Gene Therapy

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In recent years, the number of clinical trials in which adeno-associated virus (AAV) vectors have been used for *in vivo* gene transfer has steadily increased. The excellent safety profile, together with the high efficiency of transduction of a broad range of target tissues, has established AAV vectors as the platform of choice for *in vivo* gene therapy. Successful application of the AAV technology has also been achieved in the clinic for a variety of conditions, including coagulation disorders, inherited blindness, and neurodegenerative diseases, among others. Clinical translation of novel and effective "therapeutic products" is, however, a long process that involves several cycles of iterations from bench to bedside that are required to address issues encountered during drug development. For the AAV vector gene transfer technology, several hurdles have emerged in both preclinical studies and clinical trials; addressing these issues will allow in the future to expand the scope of AAV gene transfer as a therapeutic modality for a variety of human diseases. In this review, we will give an overview on the biology of AAV vector, discuss the design of AAV-based gene therapy strategies for *in vivo* applications, and present key achievements and emerging issues in the field. We will use the liver as a model target tissue for gene transfer based on the large amount of data available from preclinical and clinical studies.

AAV Vector Biology and Vector Manufacturing

Adeno-associated virus (AAV) is a small (25-nm) virus from the *Parvoviridae* family, and it is composed of a non-enveloped icosahedral capsid (protein shell) that contains a linear single-stranded DNA genome of about 4.7 kb.[1] The AAV genome encodes for several protein products, namely, four non-structural Rep proteins, three capsid proteins (VP1–3), and the recently discovered assembly-activating protein (AAP).[2] The AAV genes are required for its biological cycle and are flanked by two AAV-specific palindromic inverted terminal repeats (ITRs; 145 bp).[1] AAV viruses infect both dividing and non-dividing cells, and remain latent in the host cell DNA by integration into specific chromosomic loci (adeno-associated virus integration sites [AAVS]) unless a helper virus provides the functions for its replication.[1] AAV viruses naturally infect humans; usually an exposure to the wild-type virus occurs at around 1–3 years of age[3–5] and is not associated with any known disease or illness.[6] Importantly, the timing of human exposure to AAV viruses determines the host immunological response to the recombinant AAV vectors (*vide infra*).

In the genome of recombinant AAV vectors that are used for gene therapy, the two ITRs (viral genome *cis* packaging signals) are retained, while the other viral sequences (e.g., *rep* and *cap* genes) are exchanged with the exogenous DNA of choice. The DNA of interest flanked by the AAV ITRs is commonly referred to as the "transgene expression cassette."[7,8] Infection and transduction of cells by AAV vectors occur by a series of sequential events as follows: interaction of the viral capsid with receptors on the surface of the target cell, internalization by endocytosis, intracellular trafficking through the endocytic/proteasomal compartment, endosomal escape, nuclear import, virion uncoating, and viral DNA double-strand conversion that leads to the transcription and expression of the transgene.[9] The conversion of the AAV genome from single-stranded to double-stranded DNA occurs by both: (1) *de novo* synthesis of the complementary DNA strand (second strand synthesis), and (2) base pairing of complementary single-stranded AAV genomes derived from separate AAV viruses that co-infect the same cell (strand annealing).[9]

Differently from the wild-type virus, the genome of the recombinant AAV vectors does not undergo site-specific integration in the host DNA but mainly remains episomal in the nucleus of transduced cells, whereas random integration events are observed with a low frequency (0.1%–1% of transduction events; *vide infra*).[6,10,11] To date, 12 different AAV serotypes and 108 isolates (serovars) have been identified and classified.[1,12] The versatility of the AAV production system allows hybrid AAV vectors to be easily generated as it is composed of the same transgene flanked by the AAV ITRs from serotype 2 (the first serotype isolated and historically adopted as a gene therapy vector) and any of the available AAV capsids.[1] AAV vectors obtained through this pseudotyping method are often referred as to AAV2/n, where the first number refers to the ITRs and the second to the capsid. Because the capsid interacts with different receptors on target cells and also influences the post-entry transduction steps, AAV vectors bearing different capsids have different transduction abilities (i.e., cell tropism and kinetic of

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transgene expression), and the user can choose the most appropriate capsid to target the cell of interest.[1,13] Recently, a universal multi-serotype AAV receptor (AAVR) has been identified.[14] Because AAVR seems to be essential to AAV infection, serotype-specific co-receptors and additional factors should account for the diverse tropism of AAV capsid variants.

Previously, AAV vectors were generated from many naturally occurring serotypes.[1,13] In recent years, engineered AAV vectors, which carry novel capsids derived from rational design or directed evolution, have been generated and thereby significantly expanded the AAV vector toolkit (*vide infra*).[15]

AAV vectors can be produced at high yields by either transient triple transfection of mammalian cells,[16] infection of packaging mammalian and insect cells,[17] or other methods (reviewed by Ayuso et al.[18]). The triple-transfection method is one of the most commonly used for the production of AAV vectors, particularly in research, but also in clinical settings. It is based on the co-transfection of permissive cells (usually HEK293 cells) with three plasmids: one containing the transgene of interest flanked by the AAV ITRs, a packaging plasmid containing *rep* and *cap* genes, and a third plasmid encoding for adenoviral helper genes.[7] The purification of recombinant AAV vectors for preclinical and clinical applications is performed by either column chromatography or physical methods (gradient centrifugation).[7] Based on the purification method, the removal of both cellular debris contaminants and AAV empty capsids varies and may have an impact on the outcome of both preclinical and clinical studies.[19] One important focus in the field of AAV is to continuously improve the manufacturing processes to increase both vector yield and purity.[7,8,18,20]

Go Beyond Nature: Improve AAV Vectors by Capsid/Genome Engineering

The development of novel "synthetic" AAV vectors responds to the need for improving transduction efficiency and specificity while reducing immune recognition (*vide infra*). This is particularly important as the field has evolved from the local delivery of vectors to more systemic approaches to target entire organs (e.g., global brain delivery[21]) or the entire body (e.g., to tackle neuromuscular diseases). The increasing knowledge of AAV capsid structure-function[22] has allowed the modification of specific amino acid residues by *rational design*, while the development of AAV capsid libraries and high-throughput screening methods enabled the identification of the most efficient capsid variant for the desired cell type through *in vivo* selection (also called *directed evolution*).[15,21,23] These engineered AAV vectors are currently being evaluated in several preclinical models and could possibly substitute the vectors derived from the naturally occurring serotypes in the future.[13,24] Vector engineering has also been used to evade pre-existing humoral immunity to the AAV capsid, potentially allowing the treatment of subjects who were previously exposed to the wild-type virus. Some examples of this approach exist,[25] and hopefully clinical translation will confirm the validity of the strategy in humans.

AAV engineering that focuses on the vector genome has also been pursued. Efforts have been aimed at overcoming some of the key limitations, such as the slow onset of gene expression (due to the time-consuming conversion of single-stranded to double-stranded AAV genome) and the limited DNA cargo capacity (~5 kb). Second-strand synthesis step in AAV vector transduction can be circumvented by using self-complementary (sc) AAV vectors.[26] scAAV vectors are produced by mutating one of the two ITRs flanking the transgene so that during the AAV vector production, the Rep protein cannot solve the replication intermediates.[26] This results in packaging of "ready to express" complementary double-stranded DNA (dsDNA) vector genomes, which contain both plus and minus strands. However, due to the packaging capacity of AAV vectors, only transgenes up to ~2,400 base pairs in length could be used to generate scAAVs, significantly limiting the number of applications of this platform.[26] Notably, scAAV vectors have been demonstrated to drive faster onset and higher levels of transgene expression in a variety of tissues in animal models,[26,27] and have been successfully used in clinical trials for hemophilia B[28] and spinal muscular atrophy (ClinicalTrials.gov: NCT02122952 [29]). The small packaging capacity of AAV vectors precludes the delivery of a number of genes that exceed this size and/or the use of large physiological regulatory elements.[30] The size limitation of AAV genome can be currently bypassed by using two main strategies: oversized AAV vectors and dual AAV vectors.[30–32] Oversized AAV vectors (Figure 1A) can be generated by using large (>4.7 kb) ITR-flanked transgenes during the production AAV vector, which in turn can package genomes of heterogeneous size that are mostly truncated at around 5 kb. Once delivered to a cell via AAV vectors, the genome is reconstituted, leading to a full-length transgene expression cassette.[33] However, this step limits transduction efficiency[30] and, together with an inherent lack of homogeneity of vector preparations, represents a limitation to the clinical development of this packaging strategy.[33–36] Alternatively, dual AAV vectors are generated by splitting a large transgene expression cassette in two separate halves (5′ and 3′ ends, or head and tail); each half of the cassette is packaged in a single AAV vector (of <5 kb).[31] The re-assembly of the full-length transgene expression cassette is then achieved upon co-infection of the same cell by both dual AAV vectors followed by: (1) homologous recombination (HR) between 5′ and 3′ genomes (dual AAV overlapping vectors; Figure 1B); (2) ITR-mediated tail-to-head concatemerization of 5′ and 3′ genomes (dual AAV *trans*-splicing vectors; Figure 1C); or (3) a combination of these two mechanisms (dual AAV hybrid vectors; Figure 1D).[30] Remarkably, the use of dual AAV vectors *in vivo* results in the expression of full-length proteins and therapeutic efficacy, as demonstrated in several animal models of disease,[31,37–42] although the efficiency of these systems is still lower when compared with canonical single AAV vectors, thus requiring higher vector doses.[30,38] Nevertheless, for some applications, as seen in gene transfer directed to confined tissues such as the eye, the use of the dual AAV vector platform may represent an efficient and viable gene transfer strategy for transgenes of >4.7 kb in size.[37,38,43] Promising results are being achieved also by applying this technology to some muscular diseases,[31,39–42] and clinical development is currently being pursued for dysferlinopathy (ClinicalTrials.gov: NCT02710500).

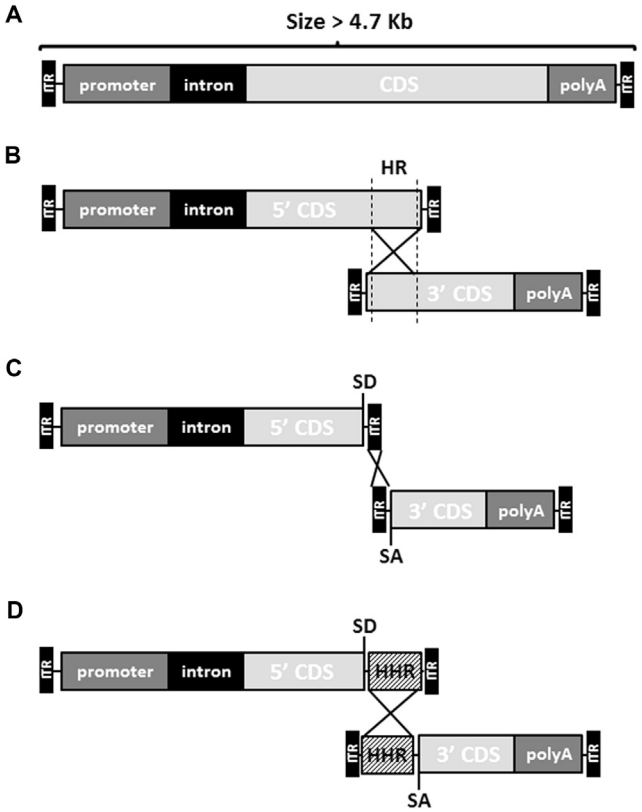

Figure 1. Schematic Representation of Oversized and Dual AAV Vector Strategies for Large Transgene Expression

Transgene expression cassettes larger than 4.7 kb can be packaged in oversized AAV vectors (A) or in regular-size dual AAV vectors that undergo genome re-assembly after cell co-transduction (B–D). (B–D) The dual AAV genome re-assembly is driven by homologous recombination between homology regions (HR) within the transgene sequence (B), inverted terminal repeats (ITRs)-mediated genome concatemerization (C), or homologous recombination between highly recombinogenic heterologous homology regions (HHR) (D). CDS, coding sequence; polyA, polyadenylation signal; SA, splicing acceptor signal; SD, splicing donor signal.

Other strategies based on the re-assembly of large proteins in the cells (e.g., protein *trans*-splicing) are also being exploited to overcome the AAV genome size restriction.[44,45] In these strategies two independent transgene expression cassettes, each encoding for a separate polypeptide/protein domain, are used and packaged in AAV.[44,45] Upon AAV gene transfer the separate polypeptides are produced and undergo re-assembly to reconstitute a full-length protein.[44,45] A similar strategy was tested in a murine and canine model of hemophilia A, in which the heavy and the light chain of coagulation factor VIII were independently expressed in hepatocytes and secreted in the bloodstream using two AAV vectors.[44,46,47]

Lessons Learned with AAV Vectors in the Clinic: Banking on the Experience with Liver Gene Transfer

The liver is a particularly attractive organ for the development of gene-based therapeutic approaches for a number of reasons including: (1) it is one of the body's major biosynthetic organs; (2) studies in small- and large-animal models and in humans have demonstrated that it is possible to target hepatocytes with adequate efficiency using intravenously administered AAV vectors;[27,48,49] (3) despite the predominantly non-integrative nature of AAV vectors,[11] multi-year transgene expression after gene transfer to the liver has been documented in large animals and humans;[28,49,50] (4) expression of a transgene in hepatocytes induces antigen-specific tolerance mediated by regulatory T cells (Tregs);[51–54] and (5) several preclinical studies demonstrate that it is possible to treat not only plasma protein deficiencies, but also metabolic disorders with liver gene transfer, resulting in long-term cure for many of these disorders in small- and large-animal models (reviewed in Kattenhorn et al.[55]).

To date, liver gene transfer with AAV vectors has been tested clinically only for a few diseases, although the landmark results obtained in the context of liver gene transfer for hemophilia B[48,49] paved the way for a number of clinical studies with AAV liver gene transfer that are ongoing. Hemophilia B is a bleeding diathesis caused by mutations in the gene for blood coagulation *factor IX* (*FIX*). Initial results in the dog model of hemophilia B provided a strong rationale for targeting the liver to express and secrete the therapeutic FIX protein.[56] In the first trial for AAV-FIX in liver, a single-stranded AAV2 vector carrying the human *FIX* transgene, which was expressed under the control of a liver-specific promoter, was administered through the hepatic artery.[57] This trial has been particularly important for the field of *in vivo* gene transfer, as it demonstrated for the first time that it was possible to transduce the human liver with AAV vectors, leading to therapeutic levels of transgene expression.[57] In addition, it identified important limitations of the approach that were related to vector immunogenicity[58] and pre-existing immunity to AAV in humans (*vide infra*). Following the results obtained in the AAV2-*FIX* trial, a second trial with intravenously administered scAAV8 vector, which encoded a codon-optimized version of the *FIX* transgene, to target the liver of hemophilia B subjects[48,49] was initiated. In this study, a short course of immunosuppression was used to block potentially detrimental immune responses triggered by the viral vector. This approach successfully demonstrated that it was possible to target the liver via the administration of an AAV8 vector delivered through a peripheral vein. Additionally, it showed that transient immunosuppression could be safely applied with gene transfer to avoid detrimental immune responses and to maintain long-term expression of the transgene product.[48,49]

Despite the small number of the clinical trials conducted thus far, the experience with liver gene transfer with AAV in humans has provided crucial knowledge on the safety and efficacy of the approach, and allowed development and testing of novel strategies to overcome immunological barriers that are posed by the human host (recently reviewed in Vandamme et al.[59]). Importantly, this knowledge would be key to achieve the goal of safe and long-term correction of various other genetic and metabolic diseases by AAV liver gene transfer.

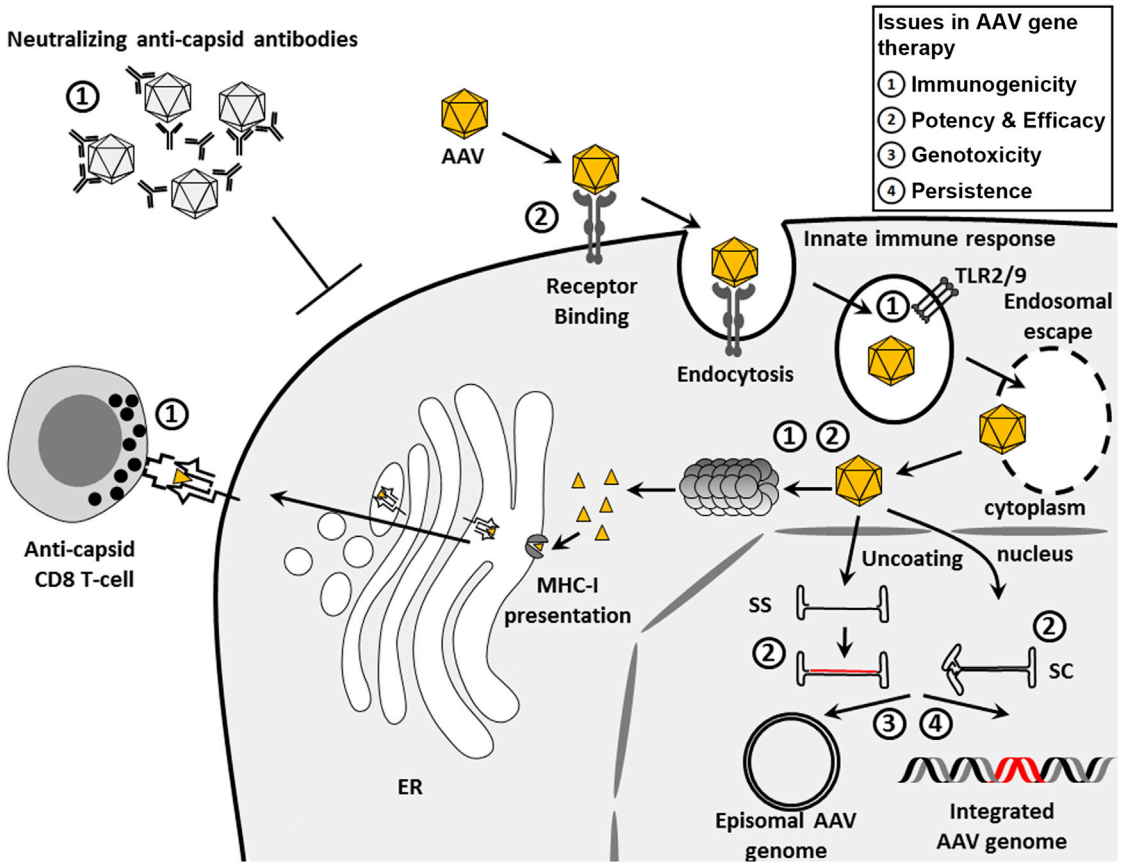

Figure 2. Current Issues in AAV Liver Gene Transfer

AAV liver gene transfer provided evidence of safety and efficacy in recent clinical trials. However, several issues related to the AAV vector platform and to the vector-host interaction are emerging (white box). These issues will need to be addressed in the future to expand the application of AAV *in vivo* gene therapy. (1) Vector immunogenicity: neutralizing antibodies (NAbs) against the AAV capsid prevent/limit cell transduction, whereas cytotoxic $CD8^+$ T cell responses eliminate AAV-transduced cells that present AAV capsid antigens loaded on major histocompatibility complex class I molecule (MHC-I) complexes. Innate immune responses contribute to the overall vector immunogenicity. (2) Potency and efficacy: the efficiency of AAV vectors at infecting and transducing the desired target cells impacts on therapeutic doses and therapeutic efficacy. (3) Genotoxicity: integration of the AAV vector DNA in the genome of the infected cell, despite being a rare event, may have genotoxic effects. (4) Persistence: because the AAV genome mainly persists in an episomal form in the nucleus of the infected cells, it can be lost in conditions of cell proliferation (such as liver growth), limiting therapeutic efficacy. ER, endoplasmic reticulum.

A number of issues still remain to be addressed before the potential of therapeutic AAV liver gene transfer in humans can be fully exploited, as discussed in the following paragraphs and schematically depicted in Figures 1, 2, and 3 and Table 1. These include: (1) immunogenicity, (2) potency and efficacy, (3) genotoxicity, and (4) persistence in a developing liver.

Immunogenicity of AAV Vectors

T Cell Responses to AAV Vectors

AAV vectors are complex biological entities, composed by both a protein capsid and a DNA genome. Each of these components may contribute to shaping the host immune response to vector-mediated gene transfer.[60] One key concept to keep in mind when studying immune responses to AAV vectors is that the viral capsid is identical or nearly identical to the capsid of the wild-type virus, to which humans are exposed.[4,61,62] Thus, it is expected that the host immune responses triggered by vector administration will be similar to those associated with a natural infection with AAV, although the quantity of viral particles administered and their route of administration may contribute to the unique features of immune responses observed in gene transfer with AAV vectors.[60]

Cell-mediated immunity directed against the AAV capsid plays an important role in terms of both safety and efficacy of AAV gene transfer in humans. This was first evidenced in a clinical trial, in which an AAV2 vector was introduced into the liver of severe hemophilia B subjects.[57] In this study, upon AAV gene transfer to liver, two subjects developed a transient and asymptomatic elevation of liver enzymes associated with loss of *FIX* transgene expression around week 4 after the vector delivery.[57] These observations were associated with the expansion of capsid-specific $CD8^+$ T cells, which likely were responsible for the immune rejection of the transduced hepatocytes.[58]

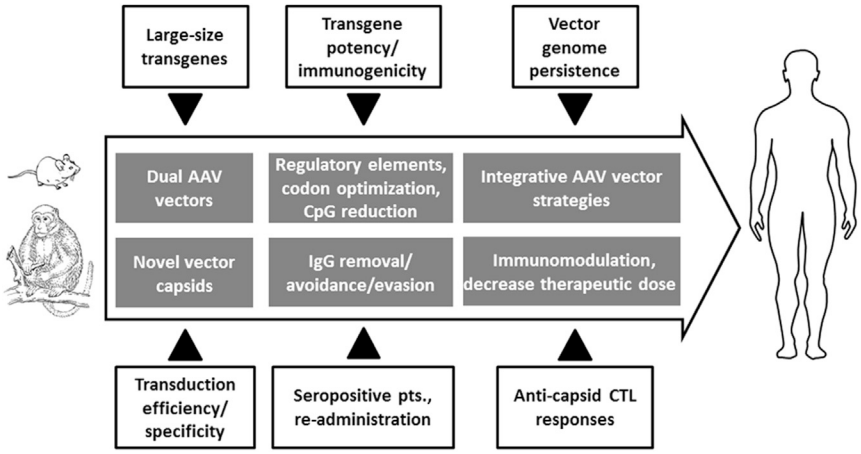

Figure 3. Schematic Path for the Optimization of AAV-Based Approaches for Human Gene Therapy
Preclinical studies performed in animal models are key to address the current limitations of AAV gene therapy approaches (white boxes) related to the vector genome and the transgene expression cassette (upper boxes) or the vector capsid (bottom boxes). Examples of various available strategies aimed at overcoming these limitations are depicted (gray boxes). pts., patients; CTL, cytotoxic T lymphocyte; IgG, immunoglobulin G.

More recently, similar observations were made in the context of a clinical trial with AAV8 gene transfer to the liver of subjects affected by severe hemophilia B.[48,49] This study showed that AAV8 vector administration in humans resulted in activation of capsid-specific $CD8^+$ T cells and increased liver enzymes in four out of six subjects from the high-dose cohort, who received 2×10^{12} vg/kg vector, ~7–9 weeks after the vector delivery.[48,49] In this study, timely intervention with oral corticosteroids was key to ablate the detrimental effect of the ongoing immune response against the AAV-transduced cells on transgene expression, and even a short delay in the administration of immunosuppression resulted in a fast and significant loss of transduced hepatocytes.[48,49] Following these two initial studies, loss of transgene expression concomitant to increase in liver enzymes was observed in several other trials (for a review, see Vandamme et al.[59] and Mingozzi et al.[63]).

One important aspect of T cell-mediated immune responses to AAV is that they seem to be detected in a dose-dependent fashion, a result consistent with published *in vitro* antigen presentation data.[64,65] Above a certain threshold of capsid antigen load, activation of capsid-specific T cells may result in hepatotoxicity and loss of transgene expression in some cases. It is not clear at this point the proportion of subjects that, after gene transfer, will develop a detrimental T cell response, associated with organ damage and loss of transgene expression. Data from the AAV8 hemophilia B trial suggest that only a subset of subjects will require immunosuppression;[48,49] however, the inherent individual differences and genetic variants (HLA type, pre-exposure to the wild-type virus, etc.) that account for the different outcome of gene transfer between subjects are still unknown. Importantly, data arising from a recent clinical trial for hemophilia B, in which subjects were treated with a scAAV8 encoding for an hyperactive FIX variant (R338L, Padua), showed the inability of immunosuppression to prevent the loss of transgene expression upon anti-capsid T cell response induction.[66] This highlights the necessity of: (1) increasing our knowledge about anti-capsid immune responses and their determinants, and (2) developing an effective immunosuppression regimen and/or other strategies to ensure stable and consistent transgene expression in humans.

The influence of vector manufacturing on the immunogenicity of AAV vectors is currently being discussed. Important open questions include the role of empty capsids, which are found in variable proportions in vector preparations. While empty particles may act as decoys for anti-AAV antibodies,[67] they may also contribute to the overall amount of capsid antigens being presented onto major histocompatibility complex (MHC) class I.[65] The presence of contaminants derived from the process used for AAV manufacturing (e.g., host cell DNA contaminants, plasmid DNA) is also a possible factor influencing the immunogenicity of AAV vectors.

The results in the AAV8 hemophilia trial by Nathwani et al.[48,49] represent an important stepping-stone in the management of unwanted immune responses in AAV gene therapy because they show that it is possible to monitor liver enzymes to design prompt interventions. However, it should be kept in mind that the ease of endpoint monitoring characteristic of this trial by the follow-ups on liver enzymes and FIX expression levels to guide intervention with steroids is unlikely to apply for all gene therapy scenarios. For example, for certain diseases in which liver enzymes are constitutively elevated, the use of immunosuppression "on demand" will not be feasible for the lack of endpoints to follow. One solution to the issue could be to administer an immunosuppression regimen up front for all subjects, as recently performed for other diseases.[29,68] However, this is not an ideal solution to the problem because not all individuals may have an immune response to the vector, and the timing of immune responses may vary according to the vector dose and serotype.[48,49,57,69] Then immunosuppression performed at the time of AAV injection may change the outcome of gene transfer by decreasing transduction efficiency[70] or may trigger unwanted reactions to the donated transgene.[52] All of these points should be carefully evaluated when designing preclinical and clinical studies.

Addressing the Issue of T Cell Responses to AAV Vectors in Preclinical Studies

Although being extremely valuable to assess AAV safety and efficacy, one major limitation of all animal models (such as mice, rats, dogs, and non-human primates [NHPs]) is that they failed to predict the issue related to the T cell reactivity to the capsid in humans. Early

Table 1. Immune Responses to AAV Gene Therapy and Possible Solutions

Immune Responses in the Human Host	Possible Solutions[a]
Anti-capsid Immunity	
Pre-existing neutralizing antibodies (NAbs) toward the capsid proteins[3,61,81]	selection of patients with low or no neutralizing antibodies[81]
	plasmapheresis[196,197]
	use of less seroprevalent capsids[61] capsid serotype switching[191–193] not-cross-reactive engineered capsids[25] exo-AAV[129] capsid decoy[67]
	prevention of NAb induction by using immunosuppressive drugs to allow AAV re-administration (if required)[195,198]
$CD8^+$ T cell-mediated cytotoxic immune response toward transduced cells presenting AAV capsid antigens	reduction of AAV capsid antigen load by decreasing therapeutic doses[149] and/or removal of empty capsids from vector preparations
	use of immune suppression (on demand or up front depending on the availability of biomarkers and endpoints, e.g., elevation of liver enzyme upon intravenous AAV administration)[29,48,49]
Anti-transgene Immunity	
Development of antibodies toward the transgene product[b]	selection of subjects having low risk of developing anti-transgene immune responses (e.g., subjects bearing missense rather than null disease causative mutations)
	use of immune suppression[198]
	use of strategies to induce immune tolerance[51,89–93,199]
$CD8^+$ T cell-mediated cytotoxicity toward the transgene-expressing cells[200,201 c]	use of immune suppression (on demand or up front depending on the availability of biomarkers and endpoints)
	use strategies to induce immune tolerance[202]
	de-targeting transgene expression from antigen-presenting cells[203]

[a]Include strategies at different stages of development (preclinical and clinical settings).
[b]Observed in animal models, not observed so far in human clinical trials.
[c]Observed so far in human clinical trials of AAV-muscle gene transfer.

mouse models in which mice were immunized against the AAV capsid were also not successful in modeling the outcome of gene transfer in humans,[71–73] leaving *in vitro* models as the only valuable tool to study capsid antigen presentation.[64,65] Recent *in vivo* mouse models of capsid T cell immune responses to AAV have been developed.[74,75] These models are quite complex but are very important for studying anti-capsid T cell immunity and to model human findings. As an example, it was demonstrated that AAV8-transduced hepatocytes remain susceptible for $CD8^+$ T cell-mediated lysis longer than those transduced with AAV2 vectors,[74] thus mimicking the observations made in human trials.[48,49,57]

From the perspective of addressing the issue on T cell immunity to AAV vectors in the clinic, one critical assessment to be made would be to evaluate the possible interaction(s) of immunosuppressive regimens applied in the context of AAV gene therapy. To this end, assessments of safety and efficacy of gene transfer combined with the proposed immunosuppression in a relevant disease model could help to identify the interactions that are potentially detrimental.[52,70]

Humoral Immunity to AAV Vectors

Following exposures to the wild-type AAV, a significant proportion of individuals develop humoral immunity against the capsid, usually starting around 2 years of age.[3–5] Maternal anti-AAV antibodies can also be found in newborns, disappearing a few months after birth and before the exposure to the virus later in life.[3–5] Thus, the temporal window in which the majority of humans appear to be naive to anti-AAV antibodies is narrow. Moreover, anti-AAV neutralizing antibodies (NAbs) can have a profound impact on the efficacy of gene transfer and should be carefully measured prior to enrollment of perspective subjects in clinical trials because of the high prevalence of anti-AAV antibodies in humans and the cross-reactivity of these antibodies across various AAV serotypes.[3–5,61,76,77]

The influence of NAbs against the AAV capsid that led to decreased vector transduction was first evidenced in the AAV2-*FIX* liver gene transfer trial.[57] In the high vector dose cohort (2×10^{12} vg/kg), one subject with a pre-treatment NAb titer to AAV2 of 1:2 expressed peak levels of *FIX* transgene of ~11% of normal, whereas another subject with a pre-treatment NAb titer of 1:17 did not have any detectable circulating FIX following vector administration.[57] These results were also confirmed by experiments in NHPs, a natural host for AAV8,[78] which showed that NAb titers as low as ~1:5 can completely block transduction of the liver following AAV8-*FIX* vector administration at doses of 5×10^{12} vg/kg.[79]

Unfortunately, anti-AAV NAb titers above a certain threshold (usually >1:5) is an exclusion criterion for subject enrollment in clinical trials requiring intravenous vector administration, because they would preclude therapeutic efficacy. Therefore, anti-humoral AAV immune response so far is a significant limitation to the application of this gene therapy platform to humans. The effects of anti-AAV NAbs and the strategies to overcome the limitations caused by anti-AAV NAbs have been summarized previously in a comprehensive discussion.[80]

Impact of Anti-AAV NAbs in the Design of Preclinical Studies

Prevalence of anti-AAV antibodies in the target patient population should be carefully evaluated when designing a gene transfer clinical trial with AAV vectors, particularly when the vector is delivered intravenously. In addition to using highly sensitive assays to measure anti-AAV NAbs,[81] preclinical studies should be used to assess the tolerance to anti-AAV antibodies based on the specific characteristics of the vector preparations such as the content of empty capsids[67] and the administered doses, because small vector doses are more prone to neutralization by NAbs to AAV. To this end, the use of *in vivo* models that were passively immunized with antibodies against AAV vectors[67,82] can be helpful because it allows consistent dosing of IgG in animals to obtain the desired NAb titers.

When designing preclinical studies in animal models, it should be kept in mind that some of them, like humans, are natural hosts for

wild-type AAVs. For instance, anti-AAV NAbs can be found in NHPs,[78] as well as in dogs and other species;[83–85] thus, pre-screening of animals for anti-vector antibodies may be required for some species and AAV serotypes.

Transgene Immunogenicity

It has been shown that AAV vector-mediated expression of human *FIX* in hepatocytes leads to tolerance to the transgene product,[51] a phenomenon that is not entirely surprising, given the unique immunological environment of the liver, which is placed at the crossroads between the gut and the bloodstream.[86] The lack of responsiveness observed in liver-directed gene transfer with AAV vectors appears to be mediated by antigen-specific $CD4^+CD25^+FoxP3^+$ Tregs,[53] which play a central role in liver-mediated tolerance induction.[52,54] Importantly, liver-mediated tolerance can be induced for various transgenes[53,87,88] and can be used to eradicate ongoing antibody responses to antigens.[89–93] The development of detrimental antibody responses to therapeutic proteins is a common complication encountered in patients affected by recessive diseases who are treated by protein replacement therapy.[94,95] Some examples of these conditions are hemophilia and Pompe disease for which patients are treated by repeated infusion of the therapeutic protein: recombinant human coagulation factors (rhFIX or rhFVIII) and recombinant human acid alpha-glucosidase (rhGAA), respectively.[94,95] Notably, AAV liver gene transfer in animal models of hemophilia[91–93] and Pompe disease[89,90] having pre-existing antibodies to the respective therapeutic proteins resulted in persistent antibody eradication. Therefore, the ability of AAV liver gene transfer to eradicate pre-existing humoral immune response to therapeutic proteins is particularly important for the treatment of these diseases.

While the immunogenicity of protein replacement therapeutics in humans is a well-established concept, relatively little knowledge is available on the immunogenicity of transgenes in humans in the context of gene therapy. To date, none of the subjects enrolled in the hemophilia trials have developed anti-transgene antibodies, despite the fact that some of them were carriers of null mutations in the *FIX* gene.[48,49,57,66] However, all subjects who had history of anti-FIX antibody formation in response to protein replacement therapies were excluded from these studies, thereby underestimating the real risk of anti-transgene antibody formation in this patient population. Future studies in larger patient populations and in previously untreated patients will help to define the risk of development of an immune response against the transgene in high-risk subjects. Of note, the vector itself, not only the transgene product, can promote transgene immunogenicity. Recent studies show that the interactions of AAV vectors with the innate immune system can have an influence on the outcome of gene transfer. In particular, they show that the AAV DNA genome and capsid structure is being sensed by Toll-like receptor (TLR) 9 and TLR2, respectively, therefore working as adjuvants for the elicitation of immune responses. AAV vectors carrying double-stranded genomes indeed showed higher immunogenicity compared with single-stranded AAV.[96–100] Recent findings also suggest that CpG sequences, ligands for TLR9, contribute to transgene immunogenicity.[101] Although these observations are limited to preclinical animal models, factors that influence transgene immunogenicity should be carefully evaluated for clinical approaches.

Assessing Transgene Immunogenicity in Preclinical Studies

Preclinical animal models of diseases provide valuable tools to evaluate the immunogenicity of gene therapeutics. One advantage of these models is that assessment of potential detrimental immune responses against the transgene product can be assayed in the context of the disease that was targeted with the gene transfer. However, several confounding factors may complicate the evaluation of transgene immunogenicity, including: (1) species specificity, because human transgenes may be highly immunogenic in other mammalians,[102–104] which may not necessarily represent the clinical scenario; and (2) genetic background, which may be associated with a complete lack of tolerance to the expressed transgene[100,105] in the cases where models were obtained by disrupting a gene, and may not be representative of the human condition. Thus, if feasible, evaluations of transgene immunogenicity should be done with species-specific transgenes and in the context of the most appropriate genetic background. Nevertheless, as a growing number of engineered transgenes are entering clinical research,[106–108] careful assessment of transgene immunogenicity in preclinical studies is important. In the case of highly immunogenic proteins, transient immunosuppression protocols can be tested in animal models of AAV gene transfer, with the caveat that some immunomodulatory drugs work only in NHPs and humans (e.g., monoclonal antibody-based immunosuppressive drugs).

Potency and Efficacy

The main objective of AAV gene therapy strategies based on gene transfer (or gene addition) is to achieve long-term stable transgene expression at levels that are therapeutic. Based on this, one prominent lesson learned from the outcome of the two AAV clinical trials for hemophilia B targeting the liver is that therapeutic levels of transgene expression can be achieved in humans in a dose-dependent manner.[48,49,57] Unfortunately, vector doses positively correlate with unwanted anti-capsid immune responses that, if not counteracted, may decrease or even abolish transgene expression (as discussed above).[48,49,57,58,69] Additionally, manufacturing of AAV vectors is complex and resource-intensive, further supporting the concept that vector optimization to achieve high potency is an important goal for the field.

Transgene expression levels required to achieve therapeutic efficacy may vary based on the inherent pathophysiology of the specific disease that is targeted and on the nature of the transgene product (e.g., intracellular versus extracellular, structural versus enzymatic function). Despite this, the design of AAV-based gene therapy strategies should always be aimed at maximizing vector potency in order to: (1) decrease the dose/response ratio and (2) reduce the risk of unwanted dose-related immune responses and toxicity, particularly when the vector is infused in the circulation. The potency of AAV for liver gene therapy can be increased by optimizing the design of

the vector (capsid and/or genome) and of the transgene expression cassette (sequence and regulatory elements). The optimization would increase either cell infection/transduction or transgene levels/activity, in turn allowing the reduction of the vector dose that is required to achieve therapeutic efficacy.

Focus on Vector Capsid, Genome, and Intracellular Trafficking

In recent years the AAV serotype 8 has emerged as the most efficient natural AAV serotype for liver transduction upon systemic delivery in preclinical models[12,28,78,109] and human hemophilia B trials.[48,49] Active research is focused on the identification of capsid variants having an efficacy profile similar or even superior to AAV8.[15] The mutations of specific tyrosine,[110] serine, threonine, and lysine[111] residues on various AAV capsids have been reported as an efficient strategy to increase the vector load that reaches the nucleus by de-targeting the viral particles from proteasomal degradation.[110,111] Improved liver transduction and faster onset of transgene expression was also reported for novel capsid variants, which were derived from *rational design*, such as the AAV2G9 generated by inserting the galactose-binding domain of AAV9 on the AAV2 capsid.[112] Another example is the "*in silico*" molecular reconstruction of ancestral AAV capsids, representing evolutionary intermediates, that lead to the *rational* generation of a novel "synthetic" vector (Anc80L65) with high tropism for many cell types, including hepatocytes.[113] High-throughput approaches employing libraries of randomly modified capsids followed by *in vivo* selection also lead to the identification of powerful "synthetic" AAV vectors.[21] One of the limitations of testing the liver transduction ability of AAV vectors in mouse (and other small-animal models) is that the results achieved may not always be extrapolated to that in the liver of large-animal models and humans. This has been recently shown for AAV2 tyrosine mutants when passing from mouse to dogs.[114] To overcome this problem, a chimeric human-mouse liver model was generated by transplanting human hepatocytes in immune-deficient $Fah^{-/-}$ mice.[115] In $Fah^{-/-}$ mice, human hepatocytes, which have a selective advantage over the murine hepatocytes, repopulate the mouse liver with variable efficiency (5%–40% repopulation efficiency). This "humanized" liver model was successfully used to screen a library of novel capsid variants and allowed to identify one chimeric capsid, AAV-LK03, derived from five different natural AAV capsids upon *DNA shuffling*, and it was able to transduce human hepatocytes more efficiently than AAV2 and AAV8.[115] The same model was also recently used to demonstrate the superior ability of an engineered AAV3 to transduce human hepatocytes when compared with AAV5, AAV8, and AAV9.[116] Still, one limitation of the model is that human hepatocytes are embedded in a non-physiological environment, including the extracellular matrix, blood composition, and the host immune system, which may influence the AAV transduction efficiency. Ultimately, the therapeutic advantage deriving from the use of novel capsid variants/mutants will have to be evaluated in the context of large-animal models, such as NHPs, and in clinical trials. The AAV vector payload to the nucleus can also be enhanced by drugs that protect the viral particles from intracellular degradation.[30,64,117–120] To date, the proteasome inhibitor bortezomib (Velcade) is a possible drug candidate to be tested in clinical trials to increase AAV-mediated liver transduction, because it is approved for human use. Interestingly, its co-administration with AAV in hemophilia B dogs resulted in 3- to 6-fold higher transgene expression in the liver.[30] However, use of this drug in the context of AAV gene transfer needs to be evaluated against the possible side effects of the drug, which include neurotoxicity.[121]

Notably, in addition to increased cell transduction, some capsid modifications and drugs may also increase AAV potency by reducing the capsid immunogenicity. For instance, the proteasomal de-targeting of AAV vectors decreases both antigen presentation on MHC class I of capsid-derived peptides[74] and the generation of NAbs that are cross-reactive to the parental serotype.[111,122] Similarly, modifications to the nature and the sequence of the vector genome have been reported to impact the AAV immunogenicity in mouse models: although scAAV genomes seem to induce a more potent innate immune responses than single-strand AAV (ssAAV),[97] the depletion of immunostimulating CpGs may prevent immune responses to both capsids and transgenes.[97,101]

The state of activation of endogenous pathways has also an important bearing on AAV transduction. Recently, autophagy activation through pharmacological inducers, such as clinically approved rapamycin, has been proven to increase hepatocyte transduction in small- and large-animal models; conversely, its inhibition resulted in decreased levels of AAV transduction.[123]

The infection of AAV target cells with unrelated viruses has recently emerged as an additional factor in influencing outcomes of gene therapy. An example of the exploitation of this physiological mechanism is represented by the enhancement of AAV transduction observed in the liver of hepatitis B virus (HBV) transgenic mice.[124]

Additional improvements in the efficacy profile of AAV gene therapy can be achieved by devising optimized vector delivery methods, as has been shown for liver[125,126] and muscle.[127] In the case of liver, studies have shown that catheterization of liver vasculature enables more efficient delivery of AAV vectors in the presence of anti-capsid NAbs.[125] Regional AAV delivery via the suprahepatic vein or hepatic artery with transient balloon occlusion of the regional hepatic venous flow also resulted in a superior AAV transduction efficiency when compared with intravenous delivery in NHPs.[126] Clinically feasible and non-invasive delivery methods (e.g., intravenous infusion via peripheral vein) still remain preferable when high transduction efficiency is not required.

Finally, vector manufacturing is expected to affect AAV infectivity and biological properties. The most emblematic example is the isolation of exosome-associated AAVs (exo-AAVs), which are naturally produced by mammalian cells during "classical" AAV production.[128] Exo-AAV have novel properties compared with non-exo-AAV provided by the envelope of cell membranes; these include reduced neutralization by NAbs, enhanced liver transduction,[129] and superior diffusion through physiological barriers such as the blood-brain

barrier.[128] Exo-AAV hold potential for the gene therapy field, although their clinical translation would need to overcome limitations related to the variability and potential toxicity of their cell-derived components.

Modifications to the vector genome, in particular, the use of scAAV vectors,[26] have shown to increase vector potency as compared with ssAAV in the liver of small- and large-animal models upon systemic delivery,[27,130–132] suggesting that hepatocytes are, to some extent, inefficient in *de novo* second-strand synthesis.[26] Notably, scAAV vectors seem to provide faster and stronger transgene expression as compared with ssAAV, allowing the reduction of vector doses while maintaining a high efficacy.[26] Strategies to increase transgene expression in hepatocytes are actively investigated in animal models. One of these strategies consists in the co-injection of a regular ssAAV encoding for the transgene of interest and scAAV vectors encoding for liver phosphatases such as T cell protein tyrosine phosphatase (TC-PTP) or protein phosphatase-5 (PP5).[133] The development of this strategy would be potentially useful to increase the expression of therapeutic transgenes that do not fit in scAAV vectors (transgene size >2.4 kb).[133]

Expression of a large transgene in mouse hepatocytes by systemic delivery of dual AAV *trans*-splicing vectors has also been reported,[134] but a proper comparison among all of the available dual AAV vector systems in the context of liver gene transfer is still missing. Approaches based on intracellular protein (rather than genome) re-assembly have also been developed to express large proteins by AAV vector.[44,45] For example, two separate AAV vectors have been generated to deliver the heavy and light chains of the large FVIII protein to overcome the AAV packaging limitation.[44] Importantly, the application of these vectors to *FVIII*-deficient small- and large-animal models resulted in correction of the hemophilic phenotype.[44,46,135]

Other modifications to the AAV vector genome can improve their efficiency, such as the elimination of sequences forming hairpin structures from the transgene sequence, which have a negative impact on AAV vector yields and lead to the encapsidation of truncated defective genomes.[136]

Focus on the Transgene Expression Cassette

In addition to capsid/genome optimization, improvements in the design of transgene expression cassettes have also been widely reported to increase transgene levels and therapeutic efficacy of AAV vectors.[101] To this aim, the design of various elements, such as transcriptional and post-transcriptional regulatory elements, guanine-cytosine (GC) content, and codon usage, can be modified and adjusted. The regulatory elements include promoter, enhancer, Kozak sequence, intron, UTRs, and polyadenylation signal.[137,138] The elimination of alternative open reading frames and cryptic splice sites in the transgene sequence also may enhance the efficiency and the consistency of transgene expression.[139] So far, the promoters used in the liver of hemophilia B patients treated in the two published AAV-based clinical trials are the hepatocyte-specific apolipoprotein E (ApoE)/human α1 antitrypsin (hAAT) promoter (consisting of human apolipoprotein E/C-I gene locus control region [HCR] combined with the human α1 antitrypsin promoter [hAAT])[140] and the LP1 promoter (consisting of core liver-specific elements from the HCR and the hAAT promoter).[27] While in preclinical settings, AAV-mediated transgene expression in the liver is reproducibly achieved using both constitutive and tissue-specific promoters; the restriction of transgene expression in hepatocytes is a preferred choice to avoid the expression of the transgene product in antigen-presenting cells (that may boost anti-transgene immune responses) and to favor induction of immune tolerance to transgene products.[51] Recently, novel hepatocyte-specific transcriptional *cis*-regulatory modules (CRMs) have been identified containing evolutionary-conserved clusters of binding sites for tissue-specific transcription factors. When the CRMs were used upstream of either strong (transthyretin [TTR]) or weak (paralemmin [Palm]) minimal liver promoters, they enhanced gene expression in mouse and in NHP liver.[141] Due to their small size, CRMs should be widely applicable by maintaining the size of transgenes within the AAV packaging limit. Codon optimization, on the other hand, increases both mRNA stability and protein translation,[142] and it has been successfully applied to increase the expression of therapeutic FIX and FVIII proteins in the liver upon AAV-mediated gene transfer.[27,106,131,143–145] Notably, the designing of a codon-optimized, engineered version of *FVIII* has achieved the therapeutic levels of transgene expression that justified the clinical development of a gene therapy for hemophilia A.[106,107,144]

Ideally, the optimization of multiple elements in the expression cassette should be performed, as seen in the example of the use of a codon-optimized and hyperactive protein variants.[93,106–108,144,146] Modified protein variants with favorable features, such as increased activity or secretion, have been developed to improve the treatment of several diseases such as hemophilia B,[93,147] hemophilia A,[106,107] and Pompe disease,[148] among others. Notably, AAV vectors encoding the human hyperfunctional factor IX Padua variant (FIX-Padua, arginine 338 to leucine)[146] and engineered FVIII variants (FVIII B domain deleted containing engineered glycosylation sequences[106]) are currently tested in clinical trials for hemophilia B (NCT02484092,[149] NCT01687608[66]) and hemophilia A (NCT02576795), respectively.[149,150] Although one of the main concerns over the use of engineered, non-native transgenes is immunogenicity, it should be kept in mind that one of the main advantages of expressing a transgene in the liver is the induction of antigen-specific tolerance,[91,92] which may in turn reduce the immunogenicity risk associated with the use of these non-native proteins.

Improving the potency of AAV vectors is a critical issue to achieve therapeutic efficacy in humans because many studies showed that ~50–100 less liver transduction is achieved in large-animal models such as NHPs compared with mouse, the most used disease animal model.[151] Based on the recent advances in vector design, it is expected that the development of optimized next-generation AAV vectors with higher potency for liver gene transfer will allow use of lower, and thus potentially safer, vector doses while maintaining efficacy and will

promote the translation of the existing proof-of-concept studies in animal models into clinical settings.

Addressing and Monitoring AAV Vector Potency and Efficacy in Preclinical Studies

One of the key steps in the development of an AAV vector-based gene therapy drug is the early selection of the combination of optimal transgene expression cassette, serotype, and viral genome for clinical use. For this purpose, it is necessary to consider key points as follows.

Rationally design the transgene expression cassette to maximize expression, for example, by transgene codon optimization.

Select the most suitable AAV genome (single-stranded versus sc [transgenes up to about 2.5 kb]; single versus dual vectors, etc.).

Select the most appropriate AAV capsid for the target cell type/disease; determine the optimal route of AAV vector administration.

Test AAV vector efficiency *in vitro* and/or small-animal models (e.g., wild-type mice by measuring viral genome copy number/cell and transgene expression (mRNA and/or protein levels and/or activity]).

Test AAV efficacy at improving and/or rescuing the phenotype of a relevant small- and/or large (preferably)-animal model of the disease (define endpoints and develop solid assays]).

Evaluate AAV vector potency doing a dose-response study in the relevant animal model of the disease using the final AAV construct chosen for clinical development.

Assess the immunogenicity of optimized vector as discussed (*vide supra*).

Genotoxicity

One main advantage of AAV vectors as gene therapy vehicles consists in the low frequency of vector genome integration in the host DNA and the low risk of related genotoxicity.[1] Despite this, the issue of AAV-related genotoxicity is essential in the context of clinical gene therapy, because random integration of vector genomes into the host DNA may lead to both loss- and gain-of-function mutations that may alter cell functionality and homeostasis. Several studies showed that the viral genomes remain mainly extra-chromosomal in the adult and neonatal mouse liver, which were transduced by AAV vectors,[140,152,153] while a minority of them integrate into the host DNA[152] with a preference for sites that are close to active genes, ribosomal DNA, and CpG sequences.[11,153–156] The potential of AAV-induced genotoxicity in the context of systemic or liver-directed gene therapy has been investigated more in depth in the recent years. To date, insertional mutagenesis by AAV gene transfer has been reported in several studies exploiting vector delivery to neonatal mice.[155,157] In particular, two pivotal independent studies showed that a systemic AAV administration to neonatal mice predisposes them to hepatocellular carcinoma (HCC)[155,157] because of the insertion of viral genomes into the RNA imprinted and accumulated in nucleus (*Rian*) locus, which encodes for many regulatory non-coding RNAs (small nucleolar RNAs [snoRNAs], microRNAs, and long intergenic non-coding RNAs [lincRNAs]). This integration leads to the dysregulation of genes (*Rlt1* and various microRNAs) flanking the insertion site promoting HCC.[155] Notably, in humans, the Rian locus is not present, although upregulation of delta-like homolog 1-deiodinase type 3 (DLK1-DIO3) locus, the human ortholog of the *Rian* microRNA locus, has been associated also with a poor survival rate in patients with HCC.[155] Interestingly, Chandler et al.[155] also showed that the preference of viral genome insertion in specific loci (such as *Rian*, *albumin*, and *α-fetoprotein*) is favored by their high transcriptional activity and positively correlates with AAV vector doses. The authors also reported that the upregulation of genes that are close to the insertion site depends on the strength of the promoter included in the transgene expression cassette.[155] In particular, the strong chicken β-actin (CAG) and (thyroxin-binding globulin) TBG promoters, but not the hAAT promoter, induced the dysregulation of gene expression that led to tumor formation.[155] Based on these findings, it becomes crucial to design and optimize the regulatory elements contained in the transgene expression cassette to find a balance between potency and possible genotoxic side effects. The tumor-initiating potential of scAAV vectors in the liver of adult mice and newborn rats has also been recently assessed.[158,159] Surprisingly, no integration hotspots of scAAV genomes were detected in the liver DNA, and no increased frequency of tumors was found in adult and newborn-treated animals.[159] However, what is still missing to date is a side-by-side comparison of the profile and efficiency of viral genome integration in the liver upon administration of ssAAV and scAAV vectors. Differently from that reported for neonatal rodents, vector administration in juvenile (6–8 weeks) or adult animals resulted in divergent observations, with studies showing no AAV genome integration[11,47,160–162] and others reporting AAV genome integration[153,163] and increased incidence of liver tumors in mice with a high physiological susceptibility to tumors.[164]

Concerns about AAV genotoxicity were recently raised upon a report on the clonal insertion of wild-type AAV2 genome sequences in human HCC liver biopsies.[165] The analyses showed insertions of AAV sequences, mostly representing fragments of the ITR, in HCC pro-oncogenes, which in turn were possibly positively selected due to the ITR transactivation effect.[166] These observations were recently corroborated by the identification of liver-specific enhancer-promoter elements in the wild-type AAV2 genome. These elements (present in a stretch of 124 nt) were found to be close to the right-hand ITR and within the 163-nt common insertion region of the AAV genome, which was previously identified in HCC biopsies.[167] Evidently, AAV vector transgenes devoid of this liver *trans*-activating genome region would be preferred for clinical use compared with those containing it.

It is worth mentioning that although some of the studies on the insertional mutagenesis of AAV vector conducted thus far in rodents revealed their potential genotoxicity, long-term studies in larger animal models such as dogs[50] and NHPs[28,168] raised no concerns over the genotoxicity risk of AAV vectors in liver. Similarly, studies in humans also support the safety of the approach, because no tumor formation

has been documented >7 years post-gene transfer in hemophilia B subjects.[49] Notably, integrational events of AAV vector genome in the proximity of HCC genes were not detected in liver biopsies from subjects who were treated with AAV5 in a recent clinical trial for acute intermittent porphyria.[168] Nevertheless, the number of AAV-treated subjects was small, and an extended period of follow-up is required. Additionally, monitoring of subjects treated with AAV at a pediatric age will help clarify the genotoxicity risk in this subject population.

Addressing and Monitoring AAV Vector Insertion and Genotoxicity in Preclinical Studies

Studies on vector integration are complex and are not strictly needed for an early-phase clinical development of gene-based therapeutics. When approaching these studies, the following key aspects should be taken into consideration.

- Use of the most appropriate experimental procedure and animal model considering, for example, model age at the time of vector delivery, model genetic background (such as wild-type, disease model, or tumor-prone animals), vector dose, and the duration of the follow-up, keeping in mind that they must be consistent with a potential clinical application.
- Monitoring of tumor formation in AAV-transduced tissues (by performing histopathological analyses after long-term observation).
- Evaluation of insertional mutagenesis of AAV vector genomes in the DNA of transduced tissues by identifying AAV vector integration sites as discussed above.

AAV Persistence in the Developing Liver

The early demonstration that AAV vectors do not integrate in significant proportion into the host liver genome comes from experiments in which partial hepatectomy was performed after gene transfer that resulted in loss of transgene expression.[169] Similarly, the transduction of actively replicating hepatocyte cells with AAV in neonatal mice leads to vector dilution over time, with transgene expression deriving from both residual episomal and integrated vector genomes.[170] For example, the intravenous delivery of a therapeutic dose of AAV vector to a mouse model of Crigler-Najjar (CN)[171] at day 2 after birth resulted in partial loss of transgene expression over time, and reduced efficiency, despite clear phenotype correction, was observed 17 months after the gene transfer.[172] Interestingly, either a short delay in the timing of AAV vector administration (approximately day 4 after birth) or increased vector doses in CN mice significantly improved the persistence of viral genomes and the expression of transgene, thus resulting in a superior therapeutic efficacy.[172] The positive correlation between AAV vector doses injected in newborn mice and increased transgene persistence has been documented in many studies.[55]

For diseases characterized by a relatively low therapeutic threshold, like CN or hemophilia (~5% of normal enzyme activity converts the phenotype from severe to mild[173,174]), a single administration of AAV vector at the appropriate therapeutic dose may be sufficient to achieve a lifelong correction in conditions of hepatocyte proliferation. AAV vector re-administration or the strategies based on viral genome integration would likely be required (*vide infra*) for the treatment of diseases that require more robust expressions of transgenes in the liver or early interventions in life.[175,176] Future clinical trials based on AAV liver gene transfer for the treatment of pediatric subjects will be key to provide new data for elucidating and addressing this issue effectively.

Differently from what was observed in neonatal animals, the transduction of post-mitotic or slowly replicating adult tissues leads to genome persistence and stable transgene expression. In dogs and NHPs treated by AAV gene transfer in the adult life, vector genomes persisted for more than 10 years mainly in episomal form with little or no evidence of genomic integration.[28,50] In humans, long-term transgene expression for >7 years after a single AAV vector administration has been shown in liver and muscle.[48]

The degree of hepatocyte proliferation and liver growth may not be easily extrapolated across species in various animal models and humans, complicating the interpretation of results and their translatability. In mice, hepatocyte number increases proportionally with liver size during the first 28 days of life,[177] while later in life, cell division occurs every ~100–200 days. Hepatocyte cell division in adult rats was instead reported to be faster,[177,178] although the fact that the comparison is made between independent studies employing different methods may account for this discrepancy. In humans, liver size increases after birth during childhood and becomes stable at approximately 10–15 years of age.[179] In addition to physiological liver growth, other factors may influence AAV genome persistence or stability following gene transfer. These include the health of the liver parenchyma (fibrotic/cirrhotic state)[180] and conditions of pathological hepatocyte turnover that are observed in several liver metabolic diseases.

To overcome the loss of AAV vector genomes in dividing tissues, such as a neonatal liver, several strategies have been tested in preclinical studies, all promoting the integration of the viral genome in the host genome. Wang and colleagues[181] included DNA homology sequences in the AAV vector genome to promote its integration in the host genome at multiple loci encoding for the 28S ribosomal RNA. This strategy significantly improved vector persistence by inducing a 30-fold increase in the vector integration.[181] The integration of *promoter-less* AAV transgenes in mouse liver by HR at the endogenous *Albumin* locus (*gene-ride* strategy) is currently exploited based on the *Albumin* strong promoter activity. Using this strategy, Barzel et al.[182] showed that therapeutic levels of *FIX* transgene could be achieved despite the general low integration efficiency (0.5% of alleles targeted). The same approach has been recently applied to neonatal CN mice, resulting in persistent transgene expression and therapeutic efficacy.[183] Due to the low efficiency of the strategies based on HR, high AAV vector doses are currently needed (~10^{14} vg/kg). Based on this, a side-by-side comparison between the HR-based integration and the regular AAV gene transfer will be

important when selecting the most effective gene delivery strategy in conditions of hepatocyte proliferation.

The stable integration of the AAV genome in the host DNA can be enhanced by combining the use of homology arms in the viral genome and of DNA nucleases that induce site-specific double-strand breaks (DBSs) to enhance HR events. This approach is usually defined as genome editing. Various nucleases can be designed and engineered to target desired DNA loci by predictable but complex protein-DNA interaction. The combination of the gene-ride strategy with liver-expressed zinc-finger nucleases (ZFNs) targeting the mouse *Albumin* locus resulted in higher levels of *FIX* transgene integration in neonatal hepatocytes and stable FIX levels in the circulation.[182,184,185] Notably, the ZFN-based gene-ride approach is currently in clinical development for the treatment of hemophilia B by targeted integration of *FIX* transgene in hepatocytes.[55] Despite current improvements, the estimated efficiency of gene transfer strategies based on HR still restricts the application of this approach to diseases that require low levels of therapeutic transgene expression. Nevertheless, these studies proved that stable integration of AAV DNA into a growing liver significantly improves the persistence of transgene expression in animal models.

Recently, the advent of the CRISPR/Cas9 system, which is an extremely versatile tool for genome editing, simplified the induction of DSB at desired loci and significantly expanded the studies aimed at promoting stable integration of the AAV genomes in the host DNA. The versatility of the CRISPR/Cas9 system relies on the use of a sequence-independent nuclease (Cas9) guided to the desired DNA sequence by a RNA molecule following canonical base-pairing interactions. The systemic delivery of Cas9 nuclease (by either AAV or non-viral methods) together with a therapeutic transgene encoded by AAV to the mouse neonatal liver led to a successful site-specific homology-driven repair (HDR) and therapeutic efficacy in models of liver metabolic diseases (e.g., ornithine transcarbamylase [OTC] deficiency[186] and hereditary tyrosinemia type I [HT-I][187]).

DNA DSBs induced by CRISPR have also been exploited to silence endogenous gene expression via physiological error-prone non-homologous end joining (NHEJ). CRISPR-mediated *in vivo* gene silencing can be used to shut off genes carrying pathological gain-of-function mutations, and thereby manipulate metabolic pathways for both therapeutic and mechanistic purposes.[188,189] More recently, Cas9 variants that have a nickase rather than nuclease activity and fused to a cytosine deaminase domain have been developed to modify a single nucleotide (C to U) in the DNA. This strategy, denominated base editing, resulted in efficient gene silencing of *PCSK9 in vivo* in the mouse liver.[190]

Although these results are undeniably promising, additional studies will be necessary to: (1) reduce the AAV vector doses that are currently required to obtain therapeutic levels of transgene integration (>1×10^{13} vg/kg); and (2) carefully evaluate the risk of genotoxic effects that are associated with off-target HR/HDR, base editing, and integration events, case by case. Insertional mutagenesis could indeed have a complete different outcome based on the integration site, cell type, cell stage, and disease-specific features, for example, tumor-prone metabolic liver diseases.

Alternative strategies that are closer to the clinical application for improving AAV persistence in a growing liver rely on the possibility of AAV vector re-administration using various approaches such as: (1) capsid switching,[191–193] (2) co-delivery of AAV with immunomodulatory molecules,[194,195] and (3) plasmapheresis.[196] All of these methodologies are aimed at overcoming the limitations imposed by the development of high-titer anti-capsid NAbs, which is induced upon the first administration of AAV vector. Notably, two different transgenes were recently shown to be produced in NHP liver after sequential administration of clinically relevant doses of AAV5ch and AAV1.[191] An effective re-administration of AAV in humans will represent a key step forward in this field.

Assessing AAV Genome Persistence in Preclinical Studies

Once a candidate therapeutic AAV vector is selected, it would be crucial to treat the animal model of the disease at different ages and vector doses, and evaluate the efficacy over time, that is, 1, 3, 6, and 12 months after the treatment. The therapeutic efficacy will likely depend on the combination of the vector potency, vector dose, and the rate of turnover of the target cells, namely all factors that are disease and tissue specific. Biodistribution studies in animals treated during neonatal stage may also significantly vary based on vector dose and administration route.

Conclusions

To date, a significant amount of critical insights into AAV vectors as tools for gene delivery have been gathered through preclinical and clinical studies. We now know that the AAV therapeutic platform has the ability to achieve an expression of a given transgene at therapeutic levels for multiple years, potentially representing a cure for chronic genetic diseases. Although safety must remain as the overarching goal for the field, achieving therapeutic efficacy in a consistent manner in adults and pediatric patients will likely be essential for gene therapies to become competitive with other approaches that are emerging as treatment modalities for genetic diseases. To meet the challenge, many aspects of AAV biological properties in the context of the human host, such as AAV vector immunogenicity, therapeutic potency, persistence, and potential genotoxicity, will have to be further elucidated. Even though preclinical animal models cannot be used to accurately predict the outcome of gene transfer in humans, they will continue to be essential for the development of highly optimized gene therapy drugs by allowing: (1) the early definition of the profile of the therapeutic product and the generation of proof-of-concept data in the relevant model(s) of the disease; (2) the determination of the optimal route of administration and its feasibility such as dose, frequency, and tissue target; (3) the optimization of the gene therapy product at early stages in the development, for example, choice of the capsid and genome conformation, to commit to a

candidate before starting the drug development; and (4) preclinical studies to find the dose for defining a robust therapeutic margin in the suitable animal model(s).

In this review, we discussed key points to outline preclinical studies that will help the design of clinical trials and the testing of safe and efficacious novel strategies aimed at overcoming the hurdles of liver gene transfer with AAV vectors (Figure 3); however, in some cases, only human studies will ultimately reveal the therapeutic value of these strategies.

CONFLICTS OF INTEREST

F.M. is an employee of Spark Therapeutics, Inc. The other authors declare no conflict of interest.

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