Expressing Transgenes That Exceed the Packaging Capacity of Adeno-Associated Virus Capsids

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Recombinant adeno-associated virus vectors (rAAV) are being explored as gene delivery vehicles for the treatment of various inherited and acquired disorders. rAAVs are attractive vectors for several reasons: wild-type AAVs are nonpathogenic, and rAAVs can trigger long-term transgene expression even in the absence of genome integration—at least in postmitotic tissues. Moreover, rAAVs have a low immunogenic profile, and the various AAV serotypes and variants display broad but distinct tropisms. One limitation of rAAVs is that their genome-packaging capacity is only ~5 kb. For most applications this is not of major concern because the median human protein size is 375 amino acids. Excluding the ITRs, for a protein of typical length, this allows the incorporation of ~3.5 kb of DNA for the promoter, polyadenylation sequence, and other regulatory elements into a single AAV vector. Nonetheless, for certain diseases the packaging limit of AAV does not allow the delivery of a full-length therapeutic protein by a single AAV vector. Hence, approaches to overcome this limitation have become an important area of research for AAV gene therapy. Among the most promising approaches to overcome the limitation imposed by the packaging capacity of AAV is the use of dual-vector approaches, whereby a transgene is split across two separate AAV vectors. Coinfection of a cell with these two rAAVs will then—through a variety of mechanisms—result in the transcription of an assembled mRNA that could not be encoded by a single AAV vector because of the DNA packaging limits of AAV. The main purpose of this review is to assess the current literature with respect to dual-AAV-vector design, to highlight the effectiveness of the different methodologies and to briefly discuss future areas of research to improve the efficiency of dual-AAV-vector transduction.

INTRODUCTION

OVER THE PAST YEARS, adeno-associated virus (AAV)-based vectors have gained rising attention as a gene delivery vehicle for preclinical research and are used increasingly in clinical trials to treat a large number of diseases. Moreover, the treatment of lipoprotein-lipase deficiency with an AAV vector is the first clinically approved gene therapy treatment in the Western world.¹ Among the most attractive features of AAV vectors are the lack of pathogenicity of wild-type AAV,^{2,*} the availability of a variety of serotypes with broad but distinct tropism, and the low immunogenicity of AAVs.

AAV is a nonenveloped, single-stranded DNA virus of the *Parvoviridae* family and was first discovered as a contaminant of adenovirus preparations.⁴ In contrast to most other members of the *Parvoviridae* family, AAV is replication defective and is only able to replicate efficiently in the presence of a helper virus such as adenovirus or herpes virus.⁵ The infectious virus particle consists of a small (20–25 nm in diameter), icosahedral capsid containing a linear, single-stranded DNA genome, approximately 4.7 kb in length, with plus and minus strands packaged with equal efficiency into preformed, empty viral particles composed of 60 copies of the three viral proteins; VP1, VP2, and VP3.^{6–8} For a recent review of AAV biology, see ref.⁵

The AAV genome organization is simple and consists only of a *rep* and a *cap* gene flanked by two

*However, a recent publication reports a possible link between the integration of AAV2 genomes into the host genome and hepatocellular carcinomas.³

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inverted terminal repeats (ITRs). During productive viral replication, the Rep proteins are needed for DNA replication as well as packaging of the viral DNA into preformed capsids.⁵ Rep proteins are also needed for the preferential integration of wtAAV genomes into human chromosome 19^{9–11} and presumably for the rescue of the integrated genome upon superinfection with a helpervirus.¹² Interestingly, even though recombinant AAVs (rAAVs) are unable to integrate preferentially into chromosome 19 (because they lack the Rep proteins), they can trigger long-term transgene expression in postmitotic tissues, most likely because they persist as largely circular episomes within the nucleus.^{13–16}

The production of rAAVs is relatively simple. The ITRs are the only *cis*-element requirement for the production of rAAVs, whereas *rep*, *cap*, and adenoviral helper genes can be provided in *trans*. Thus, rAAVs contain only the transgene DNA flanked by the ITRs, and this genome is encapsidated within a serotype-specific capsid. Although none of the available serotypes transduce only one specific tissue or cell type, collectively the 13 AAV serotypes and the myriad of AAV variants offer broad but distinct tissue tropism (see, e.g., ref.¹⁷).

PACKAGING CAPACITY OF VIRUSES OF THE PARVOVIRIDAE FAMILY

The average genome length of wtAAV serotypes is approximately 4.7 kb.^{8,18–20} This consistency in genome length is similar to the genomes of other parvoviruses, which also package single-stranded DNA into an icosahedral capsid of similar size.^{21–24} This suggests that the maximum packaging capacity of members of the *Parvoviridae* family is approximately 4.5–5.5 kb.

Numerous studies have investigated the packaging capacities of AAV. An early study²⁵ investi-

gated how much the packaging capacity of wild-type AAV could be expanded by inserting increasing numbers of 100 bp DNA fragments into wild-type AAV genomes. On the basis of these experiments, Hermonat et al. concluded that the maximum genome size that allowed the recovery of significant amounts of infectious virus was approximately 5.6 kb.²⁵ Another investigation by Dong et al.²⁶ reported that rAAVs can package genomes ranging from 2 to 5.2 kb in size. The optimum packaging size for genomes was determined to be between 4.1 and 4.9 kb, whereas vectors larger than 5.2 kb were found to be packaged very inefficiently, and transduction efficiencies of these oversized vectors were dramatically reduced.²⁶

EXPRESSING TRANSGENES THAT EXCEED THE PACKAGING CAPACITY OF AAV CAPSIDS

For most gene therapy applications the packaging capacity of AAV is not a limiting factor because the median length of a human protein is only 375 amino acids.²⁷ On the other hand, approximately 6% of all human proteins have a coding sequence that exceeds 4 kb,²⁸ which makes it difficult, if not impossible, to fit expression cassettes for these cDNAs into a single AAV genome. This group of proteins includes several therapeutic targets for a number of diseases (e.g., see Table 1).

Among the diseases caused by mutations in proteins whose cDNA size exceed the packaging capacity of rAAVs is Stargardt disease, which is caused by mutations in *ABCA4* (cDNA size ~7.3 kb). Surprisingly, in a 2008 article, Allocca et al.²⁹ reported that the packaging capacity of rAAVs is serotype specific, and that a single AAV5 capsid can encapsidate genomes as large as 8.9 kb. The authors of that study based this conclusion on the fact that rAAV purportedly encapsidating

Table 1. Examples of disorders caused by mutations in genes whose cDNA size does not allow expression from a single AAV vector

Gene	Disease	Main phenotypes
<i>Dystrophin</i>	Duchenne muscular dystrophy ^a	Progressive muscle degeneration, reduced life expectancy
<i>Dysferlin</i>	Dysferlinopathies (includes Miyoshi myopathy, limb-girdle muscular dystrophy type 2B)	Progressive muscle weakness, adolescent to young adult onset. Individuals are usually wheel chair bound in their forties.
<i>Cystic fibrosis transmembrane conductance regulator (CFTR)</i>	Cystic fibrosis	Persistent coughing, frequent lung infections, shortness of breath, gastrointestinal symptoms, male infertility, reduced life expectancy
<i>Myosin VIIA</i>	Usher syndrome 1B	Bilateral deafness from birth, decreased night vision starting in early childhood, balance problems
<i>ABCA4</i>	Stargardt disease	Severe vision loss, legal blindness ^b
<i>Factor VIII</i>	Hemophilia A	Impaired blood clotting, increased risk/duration of bleeding, reduced life expectancy

^aBecker's muscular dystrophy is caused by mutations in the dystrophin gene as well but has a milder phenotype than Duchenne muscular dystrophy.

^bDefined in the United States as visual acuity of 20/200 or worse.

the ~7.3-kb-long cDNA for *ABCA4* (plus promoter, poly A signal, and ITRs) successfully transduced murine retinas and improved the ocular phenotype in *ABCA4*^{-/-} mice.

However, the finding that AAV5 capsids could package intact genomes of up to 8.9 kb in length was not supported by further studies, which found that such genomes were truncated and that the packaging capacity of AAV was still limited to genomes substantially smaller than 8.9 kb.^{30–32} In fact, these reports confirmed earlier studies that the packaging capacity of AAV is roughly 5 kb. In one study by Lai et al.,³⁰ the authors investigated expression from AAV5 vectors with an oversized genome (~8.2 kb) that, if intact, would allow the simultaneous expression of alkaline phosphatase (AP) and β -galactosidase (LacZ). But although Duan and colleagues could detect the concurrent expression of both AP and LacZ, further molecular characterization found that the oversized virus stock was in fact a mixture of partially packaged viruses. This finding was supported by Wu et al.,³² who found that packaged vector genomes never exceeded 5.2 kb in length, and that plasmid encoded vectors exceeding 5.2 kb existed as a mixture of genomes that were heterogeneous in size. Thus, it is hypothesized that packaging of oversized genomes into preformed AAV capsids leads to the truncation of the unpackaged length of genome by cellular nucleases, leading to fragmented AAV vectors (fAAVs). As the packaged genomes are of varying length and polarity, transduction of a single cell with two truncated fragments may lead to hybridization of any overlapping segments, which are then repaired by the host-cell DNA replication/repair machinery to form a full-length transgene.^{33,34}

SHRINKING THE SIZE OF THE TRANSGENE CASSETTE BY MINIMIZING THE SIZE OF REGULATORY ELEMENTS AND SPLITTING OR TRUNCATING THE THERAPEUTIC PROTEIN

In addition to the cDNA of the therapeutic gene and the ITRs, additional elements need to be included into the genome of rAAVs encoding a therapeutic protein. At a minimum, these include a promoter and a polyadenylation signal. Because most tissue-specific promoters are large, and minimal versions of such promoters often lose tissue specificity and/or reduce transgene expression, most AAV vectors encoding large therapeutic proteins use the strong, constitutively active CMV promoter, which is ~600 bp in length. However, it has been reported that the CMV promoter can be

shortened to 173 bp without apparent loss in expression levels—at least in certain cell types.³⁵ The most widely used polyadenylation sequence, the late SV40 poly A sequence, is approximately 300 bp in length. But it has been reported by McFarland et al. that the 32-bp-long polyadenylation of soluble neurophilin 1 is equally efficient in mRNA polyadenylation when compared with the late SV40 polyadenylation signal,³⁶ thus allowing a reduction of an rAAV genome size by ~270 bp.

Nonetheless, the use of short promoters and polyadenylation signals is clearly insufficient to allow the encoding of some full-length therapeutic proteins in a single AAV genome. Hence, different strategies, namely, splitting or truncating the therapeutic protein—while retaining its functionality—have been attempted for several proteins of interest for gene therapy. One example of successfully splitting a gene while retaining its functionality is factor *VIII* (*FVIII*), mutations in which are the cause of hemophilia A. FVIII is produced as a 256 kDa precursor protein, which is processed within the cell into an approximately 200 kDa heavy and a ~80 kDa light chain.³⁷ Both the heavy and light chains of FVIII circulate as inactive precursors in the blood. Hence, Burton and colleagues decided to express the light and heavy chain from two separate AAV vectors.³⁸ The light chain can easily be accommodated within a single AAV vector. But the full-length heavy chain is still too large to be expressed from a single AAV vector. Therefore, Burton et al.³⁸ generated two AAV vectors: one vector encoding a truncated, but functional, heavy chain and a second vector encoding the light chain. They then demonstrated that co-injection of the two vectors into C57/BL6 mice resulted in the production of therapeutic levels of biologically active FVIII. More recently, Hu and Lipshutz used a similar dual-vector strategy to demonstrate phenotypic correction of FVIII deficiency in a mouse model of hemophilia A.³⁹

An example of successfully truncating a gene was reported by Ostedgaard et al.³⁵ In this study, Welsh and colleagues used a minimal CMV promoter but also truncated the cystic fibrosis transmembrane conductance regulator Cl⁻ channel (CFTR), which has a cDNA of 4,450 bp. CFTR was shortened by a partial deletion of the R domain from the N-terminus, thus reducing the genome space required within the rAAV vector. Transduction of airway epithelial cells with an rAAV vector containing this expression cassette was sufficient to reverse, at least partially, the cystic fibrosis chloride transport defect in these cells.³⁵

Dystrophin, whose (partial) loss of function is the cause of Duchenne and Becker muscular dystrophy, is another example where truncated, yet functional protein variants can be generated. In fact, the so-called microdystrophin variant can be packaged in a single AAV vector, and in *mdx* mice (a mouse model of Duchenne muscular dystrophy) gene transfer with AAV vectors encoding microdystrophin provides significant, although not optimal therapeutic benefit.^{40–42}

However, for other diseases the generation of truncated versions that retain protein function has so far not been achieved. Consequently, the development of additional methods to express large therapeutic proteins by AAV gene transfer remains an important area of research. One approach that has gained considerable attention is the delivery of large genes from two, or even three, AAV vectors.

SPLIT VECTOR SYSTEMS TO EXPRESS PROTEINS THAT CANNOT BE DELIVERED WITH A SINGLE AAV VECTOR

Fragmented vectors

It had previously been claimed that single AAV vectors purportedly encapsidating genomes of up to 8.9 kb were capable of transducing cells.²⁹ However, shortly thereafter it was shown by multiple groups that AAV could, in fact, not package genomes of 8.9 kb.^{30–32} Nonetheless, AAV5 vectors with the supposedly 8.9 kb large viral genomes clearly did result in expression of ABCA4, MYO7A, and CEP290 from genomes whose *intact* genome length would be 8.9, 8.1, and 8.9 kb in size, respectively.

A potential explanation for this apparent paradox was provided by Duan and colleagues in 2010.³⁰ Duan's group constructed an oversized AAV5 vector with an AAV genome of approximately 8.2 kb in length that contained both a 5' AP and a 3' LacZ expression cassettes.

As would be expected, in a slot blot with probes directed against the AP and the LacZ open reading frames (ORFs), both probes hybridized to viral DNA from purified rAAV. Moreover, both AP expression and LacZ expression were detected in *mdx* muscle of adult mice injected with such AAV vector preparations. Critically, however, no full-length viral genomes of ~8.2 kb could be detected by alkaline gel Southern blot.³⁰ Instead, Southern blot hybridization revealed that purportedly oversized packaged genomes were in fact truncated, and the authors suggested that the truncated fragments could assemble thus restoring the full-length expression cassette. In addition, a report by Wu et al.

also found that packaged AAV genomes never exceeded 5.2 kb.³²

Overlapping dual vectors

It was first reported in 2002 that simultaneous infection of cells with two vectors containing an overlapping fragment of 440 or 1000 bases of the AP gene could successfully transduce HT-1080 cells, although only at 1–2% efficiency of a vector that expressed AP from a single vector genome.⁴³ However, in mice, nasal aspiration of AAV6 with either the overlapping vector pair or a vector encoding AP from a single genome yielded better results compared with the *in vitro* experiments. *In vivo*, the best overlapping vector pair yielded almost identical transduction of the airway epithelium when compared with the single-genome virus. As expected, when the authors compared different vector pairs, they could detect transduction only with vector pairs that shared either 440- or 1000-base regions of homology but not with vector pairs without homology.⁴³

This overlapping vector approach has been exploited by several researchers seeking to express large transgenes from AAV vectors. For instance, the overlap method was also used successfully with a split mini-dystrophin expression cassette with a combined length of approximately 7.3 kb and a short, 372 bp overlap region.⁴² In that study, expression of mini-dystrophin was observed in striated muscle of *mdx*-mice when rAAV6 was given intravenously. Although the overall levels of mini-dystrophin expression were lower than in wild-type muscles, there was an improvement in limb muscle physiological performance.⁴²

Sondergaard et al. provided another example of the successful use of an overlapping vector system to overexpress genes that exceed the packaging capacity of a single AAV vector.⁴⁴ In this study, the authors found high levels of muscle transduction with two overlapping AAV vectors encoding a *dysferlin* expression cassette (*dysferlin* has a cDNA size of 6.5 kb and the overlap between the two vectors was ~1 kb).⁴⁴ In yet another study, overexpression of dysferlin by intramuscular injection of AAV8 encoding dysferlin from either a single oversized vector or from two overlapping vectors showed a ~10-fold higher expression of dysferlin with the dual-vector system.⁴⁵ Similarly, Trapani et al. found the overexpression of MYO7a in HEK293 cells with AAV2 vectors to be more efficient when they used a dual overlapping vector system as opposed to a single oversized vector,⁴⁶ and Dyka et al. demonstrated that the expression of MYO7A in photoreceptors was stronger with a

dual overlapping vector system when compared with a single oversized vector.⁴⁷

However, the successful use of dual overlapping vectors has not proven equally successful in all cases. For example, in contrast to Dyka et al.,⁴⁷ a study investigating the efficacy of AAV2 and AAV5 vectors to deliver MYO7A, mutations in which cause Usher syndrome 1b, to mouse retinas found the dual overlapping vectors to be less efficacious than a single vector with a genome of ~7.9 kb in size.⁴⁸ The reasons for such differences in transduction efficiency are incompletely understood. But among other parameters it has been reported that the nature of the transgene⁴⁷ and tissue/cell type (possibly because of differences in DNA repair pathways)^{45,46} can influence the efficiency of dual vectors. Furthermore, the combination of

AAV serotype and tissue/cell type might have a particularly profound effect on the efficiency of transduction with overlapping systems because for *dual-vector* approaches to be successful, a high level of co-transduction—preferably with multiple copies of each vector—is essential for efficient transgene overexpression.

Moreover, even such seemingly arcane technical details as the method of vector titration appear to be able to influence the results obtained when comparing two different strategies to increase AAV payload.⁴⁶

The mechanism of transduction by fragmented and overlapping vectors is as yet not fully understood. But it is generally believed that some form of recombination occurs between the two partial genome components (Fig. 1A). Nevertheless, whereas

Figure 1. Schematic illustration of the overlapping and trans-splicing mechanisms to express genes that exceed the packaging capacity of a single AAV vector. **(A)** Reconstitution of oversized transgene expression cassettes from two vectors with partially homologous genome sequences. In this mechanism, the first vector contains the promoter and the 5' half of the coding sequence and the second vector contains the 3' half of the coding sequence and the polyadenylation signal. The 3' region of the coding sequence of the first vector and the 5' region of the second vector are homologous. A full-length cDNA expression cassette that includes all the regulatory elements is then reconstituted through homologous recombination of the overlapping regions of the two vectors. **(B)** Expression of full-length, oversized transgene cassettes through so-called trans-splicing. In this approach, the first vector contains the promoter and the first exon followed by a splice donor sequence (SD). The second vector contains a 5' splice acceptor sequence (SA) followed by the second exon and a polyadenylation signal. After uncoating and second-strand synthesis, AAV genomes form linear or circular, mostly head-to-tail, concatamers. This concatamerization of a right-hand ITR (shown in red) from the first vector with a left-hand ITR (shown in blue) from the second vector reconstitutes a full-length transcription cassette. Transcription then generates a pre-mRNA that contains the entire cDNA interrupted by an ITR-intron (dashed magenta box) followed by a poly A tail. Splicing leads to the removal of the ITR-intron and the formation of the full-length, mature mRNA. Color image available online at www.liebertpub.com/hgtb

the homologous recombination that occurs during meiosis or in double-stranded DNA break pair has been studied in great detail, the same is not true for the mechanism of the putative homologous recombination taking place in dual-vector reconstitution to a single genome.⁴⁹ But, interestingly, Samulski and colleagues reported that transduction by oversized vectors depends on the activity of the strand-annealing protein Rad51C but is independent of the catalytic subunit of DNA-dependent protein kinase.³⁴ Future research in this area is clearly important because mechanistic insight into the process of full genome reconstitution from single oversized vector and dual-vector systems will likely help in designing improved dual-vector systems.

Dual vectors: concatamers and trans-splicing

An alternative approach for splitting a genome across two vectors is to take advantage of the natural concatamerization ability of AAV. During transduction, rAAV genomes have been shown to form circular or linear (mostly) head-to-tail arrays of viral genomes,^{14,50,51} and it is generally believed that rAAV genomes persist episomally as mostly circular concatamers¹⁶ in postmitotic cells, leading to long-term transgene expression.

This concatamerization ability of AAV has been exploited in the development of dual-vector rAAV systems, in a technique called trans-splicing. Trans-splicing involves the splitting of a gene expression cassette into two parts, each packaged into separate rAAV vectors (Fig. 1B). The first vector encodes the promoter and a 5'-fragment of the ORF of the protein to be expressed, followed by a splice donor site. The second vector starts with a splice acceptor site followed by the 3' segment of the ORF of the protein to be expressed and a poly A signal. When both vectors co-transduce a cell, the viral genomes form heterodimers and concatamers presumably via intermolecular recombination/ligation⁵⁰ (Fig. 1B).

The trans-splicing method to expand the size of ORFs that can be successfully expressed using AAV vectors was originally developed in the laboratory of John Engelhardt^{52,53} and has since been utilized in several studies with varying success (e.g., refs.^{53–57}).

Interestingly, similar to dual overlapping vectors, trans-splicing vector-pair systems do not always outperform single oversized vectors. For instance, Hirsch et al.³⁴ reported higher expression both *in vitro* (C2C12 myoblasts and myotubes) and *in vivo* with a single oversized

genome vector when compared with a trans-splicing system.³⁴

In this context, it needs to be pointed out that for all dual-vector systems (and oversized, i.e., fragmented vectors) to be successful—at a minimum—a cell must be co-infected by AAVs encoding the 5' and 3' part of the expression cassette, respectively. Moreover, the efficiency of reconstituting a genome with a full-length expression cassette is likely dependent on the number of nuclear copies of vector genomes.

For overlapping vector systems, nuclear import and uncoating must then be followed by homologous recombination of the 5' and 3' parts of the expression cassette. Conversely, for trans-splicing vector systems, and for hybrid vector systems (in the absence of homologous recombination), co-infection of a cell by AAVs encoding the 5' and 3' portion must be followed by appropriate concatamerization of the two vectors. This can be a limiting step for transduction. One of the reasons is that AAV vectors with two complementary ITRs can form circular concatamers.^{13–16} Because such a circularization is an *intramolecular* event, it is probably more efficient than *intermolecular* concatamerization.^{34,49,58} Moreover, of the 10 possible two-vector linear concatamers, only one has the correct tail to head orientation. Consequently, it is likely that the formation of “correct” concatamers of two, or more, AAV genomes increases as the copy number of AAV genomes in the nucleus rises. To address the potential limitations imposed by the need for correct concatamerization, Engelhardt and colleagues designed AAV vectors with heterologous ITRs, for example, the vector encoding the promoter and the first exon carried a left-hand AAV5 and a right-hand AAV2 ITR, whereas the vector carrying the second exon and the poly A signal carried a left-hand AAV2 and a right-hand AAV5 ITR. As predicted, intramuscular injection of a vector pair with heterologous ITRs resulted in a 3–6-fold higher expression level when compared with a homologous vector pair.^{49,59} In contrast, Auricchio and colleagues did not observe a significant increase in full-length ABCA4 production when they compared homologous and heterologous ITR vector pairs in HEK293 cells.⁶⁰

However, head-to-tail concatamer formation is not the only factor that can limit full-length transgene expression with trans-splicing vectors. A mandatory step for the successful expression of proteins from two trans-splicing AAV vectors is that splicing of the pre-mRNA must occur across the ITR junction (Fig. 1B). Lai et al. hypothesized

that this “trans-splicing” event might be rate limiting, and that a rational selection of an exon-splitting site could improve the transduction efficiency.⁶¹ They tested this hypothesis by using a mini-dystrophin gene split across two vectors and by testing the three exon/intron/exon sites that showed the highest splicing efficiencies as determined by RNase protection assays.⁶¹ Intramuscular injection of AAV6 vectors with different exon/intron/exon splitting sites demonstrated that the nature of the splice site substantially influences transduction efficiencies, and the authors could demonstrate that with the optimal exon/intron/exon combination approximately 80% of myofibers expressed mini-dystrophin.⁶¹ The importance of the nature of the splice site was further stressed in a later study by the same authors,⁶² where a synthetic intron was developed in order to try to overcome the labor-intensive process of intron screening. In this study, Lai et al. used the same mini-dystrophin construct but with an artificial intron. They could demonstrate improved expression with vectors containing this artificial intron when compared with trans-splicing vectors that contained a poor splice site; however, expression was not as efficient as with the best trans-splicing vectors using natural dystrophin exon/intron sites.⁶² Confirming these results, recent publications have shown greater success when the researchers identified the optimal exon-splitting site in the design of dual trans-splicing vectors with other transgenes. One such study, which investigated the use of *dysferlin* transgenes in the treatment of dysferlinopathies, exploited the concatamerization ability of AAV and by splitting the 6.2 kb cDNA between exons 28/29.⁶³ A/J mice, which do not express functional dysferlin,⁶⁴ were injected intramuscularly with dual rAAV vectors, encoding the 5′ and 3′ parts of the dysferlin expression cassette, respectively. As expected, successful trans-splicing resulted in the expression of full-length dysferlin, leading to improved membrane repair capacity, muscle histology, and muscle function.⁶³

Hybrid dual-vector systems

Development. The nomenclature as to what constitutes a hybrid-AAV vector is confusing because, depending on the article, it is used for two different vector systems. In some articles it refers to AAV vectors that have ITRs from two different serotypes (e.g., the left ITR from AAV2 and the right ITR from AAV5). More commonly, hybrid AAV vectors refer to dual-AAV-vector systems in

which the mechanism resulting in the transcription of full-length mRNA can occur through recombination of homologous regions or/and trans-splicing. For the purpose of this review, we will use the latter definition.

Hybrid vector systems were originally developed by Ghosh et al. in the laboratory of Dongsheng Duan.⁶⁵ Duan and colleagues hypothesized that hybrid systems should result in more efficient transgene expression because the expression of full-length protein could be achieved through either overlapping/homologous recombination or through trans-splicing. To test this hypothesis, Ghosh et al. compared expression of LacZ from an expression cassette that was either packaged in a single vector, or split across two vectors based on the overlapping strategy, two vectors based on the trans-splicing strategy, or a hybrid two-vector system that would allow both trans-splicing and homologous recombination between overlapping regions of the two vector genomes⁶⁵ (Fig. 2).

For the hybrid system, one vector contained a CMV promoter, the 5′ portion of the *LacZ* gene, and a splicing donor signal, followed by a highly recombinogenic 872 bp *AP* gene fragment. The other vector contained the same 872 *AP* fragment, followed by a splice acceptor signal, the 3′ portion of the *LacZ* gene, and a poly A signal. The choice of the 872 bp *AP* fragment was based on earlier work that showed that this *AP* fragment is highly recombinogenic in the overlapping vector system.⁶⁶ The two vectors of this hybrid system can lead to the transcription of full-length mRNA by one of two ways: either through *AP*-mediated homologous recombination or via ITR-mediated head-to-tail concatamerization (Fig. 2). Either pathway results in LacZ expression.

The efficacy of the hybrid vectors was assessed both *in vitro* and *in vivo*. When MO59K cells were transduced with the hybrid vectors at a multiplicity of infection of 20,000 viral genomes per cell (10,000 vector genomes of each vector), the LacZ expression from the hybrid vectors was equal to that of the single intact vector. Even when only 1,000 vector genomes per cell were used, the transduction efficiency reached 71% of the single vector with a genome size fitting into a single AAV capsid.⁶⁵ Thus, the hybrid vector outperformed both the overlapping and trans-splicing variants, which only expressed levels of approximately 25% and 50%, respectively, of the original construct. To confirm that the hybrid vectors could transduce skeletal muscle, viral vectors were injected into the tibialis anterior muscles of 6–8-week-old BL10 mice.

Figure 2. Schematic illustration of the hybrid dual-vector mechanism to express genes that exceed the packaging capacity of a single AAV vector. The hybrid approach combines the mechanisms of both the trans-splicing (**A**) and the overlapping (**B**) techniques. In this system, the 5' half of the first vector contains the promoter, the 5' half of the coding sequence, a splice donor sequence, and a region (shown in pink) that is homologous to a region just after the left-hand ITR of the second vector. The homologous region in the second vector is followed by a splice acceptor sequence, the 3' half of the coding sequence, and the polyadenylation signal. The splice donor and acceptor sequences and the homologous region of the two vectors can be from an intron that occurs naturally in the gene to be expressed, or the intron can consist of an artificial, highly recombinogenic region flanked by splice donor and acceptor sequences. As shown in (**A**) head-to-tail concatamer formation allows the generation of a full-length mature mRNA via trans-splicing. Similarly, homologous recombination between the natural or artificial introns also allows the formation of a full-length mature mRNA (**B**). Color image available online at www.liebertpub.com/hgtb

Expression from the single vector with a “regular size genome” and the hybrid vectors was strong and widespread, whereas expression from the trans-splicing, and especially the overlapping vectors, was not as effective. When β -galactosidase activity from muscle tissue was quantified, the hybrid vectors yielded 81% of the activity of the single “regular size genome” vector, which was significantly greater than the β -galactosidase activity from overlapping (34%) or trans-splicing vectors (62%).

These promising results notwithstanding, it needs to be pointed out that hybrid AAV vectors are

not always the best available vector system to overexpress genes that exceed the packaging capacity of a single AAV capsid (see below).

Optimized recombinogenic sequences. The study by Ghosh et al.⁶⁵ provided an important proof-of-principle of the recombinogenic ability of the *AP* gene. However, the size of the *AP* sequence (0.87 kb within each vector) was a burden on the packaging capacity of the two vectors, effectively reducing the combined genome capacity of the dual-vector system from 10 to 8.2 kb.⁶⁵ To address

this potential limitation, Duan and colleagues performed a detailed study to determine whether the *AP* bridging sequence could be shortened while still retaining efficient homologous recombination.⁶⁷ Using this approach, the authors were able to demonstrate that the homologous regions on both vectors could be reduced to 0.27 and 0.26 kb, respectively, without apparent loss of transduction efficiency when compared with the original hybrid vectors (with homology regions of 0.87 kb). Thus, these reduced bridging sequences allow for the expansion of the transgene payload by ~1.2 kb in a dual hybrid AAV vector system.⁶⁷ Even shorter, 77-bp-long homology regions (HR), derived from the filamentous phage F1, were described by Auricchio and colleagues.⁴⁶ When the authors compared three minimal *AP* HR constructs to the phage F1 HR constructs in HEK293 cells, one of the shortened *AP* HR vectors (AP1) showed slightly higher expression levels compared with the two other vector systems.⁶⁰ On the other hand, *in vivo*, *ABCA4* expression in photoreceptor cells occurred more frequently in mice injected subretinally with F1 HR vectors when compared with mice injected with *AP* HR vectors.⁶⁰

Therapeutic applications. To assess the therapeutic utility of this hybrid system with recombinogenic regions of reduced size, Zhang et al. tested these novel, hybrid vectors in a mouse model of Duchenne muscular dystrophy.⁶⁸ In that study, a *dystrophin* mini-gene was utilized that contained the neuronal nitric oxide synthase (nNOS) binding domain,^{68,69} which was expected to improve the therapeutic efficiency compared with previous mini-*dystrophin* genes by restoring sarcolemmal nNOS. They used four sets of dual vectors: sets 1–3 contained constructs that were based on the hybrid system that included the minimized *AP* bridging sequences.⁶⁷ The fourth dual-vector set was based on an overlapping strategy with an approximately 0.4 kb overlapping region of the *dystrophin* gene shared by both vectors. Interestingly, they found that muscle transduction in the *mdx4cv* mouse model of Duchenne muscular dystrophy was significantly greater when the dual overlapping vector system was used—with an expression level approximately three times higher than with vectors based on the hybrid approach. These results provide further evidence that the choice of the best dual-vector design can depend significantly on the nature of the therapeutic protein.

Moreover, the homologous recombination efficiency of particular artificial recombinogenic se-

quences can vary drastically depending on the specific applications. For example, a study by Trapani et al.⁴⁶ investigated the efficacy of dual hybrid vectors for the treatment of inherited retinal degenerations. To determine which split vector system would be most efficacious in the transduction of large genes in the retina, the authors compared oversized, overlapping, trans-splicing, and hybrid vectors with *EGFP*, *ABCA4*, or *MYO7A* as transgenes. For the hybrid vectors, the authors used both the shortened *AP* recombinogenic regions described by Ghosh et al.⁶⁷ and a second hybrid dual-vector system that contained a recombinogenic region based on a 77 bp sequence from an F1 phage genome in place of the *AP* fragment, termed dual AAV hybrid-AK.⁴⁶ In their study, Trapani et al. demonstrated that *in vitro* transduction with vectors with oversized genomes did not result in efficient transgene expression.⁴⁶ But, interestingly, they also found that the vectors using the hybrid *AP* system expressed very poorly when compared with overlapping, trans-splicing, and hybrid-AK vectors.⁴⁶ Based on these results, the authors decided to use the trans-splicing and hybrid-AK vectors to transduce mouse photoreceptors and retinal pigment epithelium in order to rescue the phenotype in *ABCA4*^{-/-} and *sh1* mouse models of Stargardt's disease (STGD; Table 1) and Usher syndrome type 1B (USH1B; Table 1), respectively. Consistent with their *in vitro* results, dual trans-splicing and hybrid-AK vectors successfully transduced mouse and pig photoreceptors and were found to improve the phenotype of mouse models of STGD and USH1B.⁴⁶

Interestingly, however, both Dyka et al.⁴⁷ and Trapani et al.⁴⁶ observed the expression of both full-length and smaller fragments (of defined size) of *MYO7A* and or *ABCA4* with the hybrid dual-vector system. Not only are such fragments likely nonfunctional, but they also could be dominant-negative and/or toxic. A potential solution to this challenge was provided by Auricchio and colleagues, who showed that the inclusion of an in-frame degron after the splice-donor site (for the 5' vector) prevents the accumulation of such fragments.⁶⁰

Taken together, these results provided further evidence that hybrid dual AAV vector systems are a promising platform to express large genes with AAV vectors. But they also demonstrate that there is no one-fits-all dual-vector system, and that the nature of the artificial recombinogenic regions and the tissue being targeted can drastically influence the efficiency of transduction with such hybrid split AAV vector systems.⁴⁶

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The literature surrounding the efficacy of dual-vector approaches is broad and often conflicting. Nonetheless, overall—if carefully optimized—the hybrid vector approach, which combines the overlapping sequence and trans-splicing methodologies, appears to be, at least for now, the most promising approach in most instances. In this context, the most important components of a dual hybrid vector system to be taken into account appear to be the choice of the recombinogenic region and the nature of splice donor and acceptor signals. If natural exon/intron/exon boundaries are used, this requires the testing of several exon/intron/exon combinations for the efficiency of expression.

It needs to be pointed out, however, that hybrid systems are not always the most efficient vector systems available. For instance, Zhang and Duan demonstrated that a simple, dual overlap vector system outperformed three independent hybrid systems,⁶⁸ and Pryadkina et al. obtained similar results when they analyzed the efficiency of dysferlin overexpression obtainable with dual overlapping vector or hybrid vector systems.⁴⁵

Despite the current limitations of dual-vector systems, recent research has investigated the possibility of expanding the dual-vector system to a triple-vector system—expanding the genome capacity of AAV vector systems even further. For example, Koo et al.⁷⁰ created a triple-vector system based on the trans-splicing approach that was capable of delivering the full-length 11.1 kb *dystrophin* genome via three vectors. In order to enhance the formation of the correct head-to-tail concatamers, the left and right ITRs differed in two of the three vectors, one ITR being an AAV2 ITR and the other an AAV5 ITR (the ITRs of the third vector were both AAV2 ITRs).^{59,60} Although full-length dystrophin could be detected with this three-vector system—both *in vitro* and *in vivo*—the expression was very weak. Nonetheless, future optimization of this technique may allow the use of AAV gene transfer for proteins that are too large for even dual-vector systems.

Maybe the firmest conclusion that can be drawn from the vast trove of available literature is that there is no one ideal system, and that the choice of what the best vector system is can vary drastically depending on the transgene and the cell type/tissue targeted by the AAV vectors. Thus, with the currently available vector systems, to achieve optimal results, researchers must optimize the vector(s) for each transgene and tissue type being targeted.

In conclusion, with further improvements dual AAV vector systems for gene therapy might allow the delivery of larger proteins and/or the inclusion of tissue-specific promoters and regulatory elements into AAV gene transfer delivery vehicles, thus allowing a wider repertoire of genetic disease to be targeted.

So far, however, the efficiency of transgene expression from dual-vector systems has been inconsistent. At present, for many clinical gene therapy applications, the efficiency of expression from the available dual-vector systems is insufficient. A better understanding of the mechanisms that limit the efficiency of trans-splicing and/or homologous recombination in hybrid vectors will hopefully lead to vector systems that allow greater transgene expression. However, the possibility of further improvements of the hybrid vector systems will eventually reach its limits. Consequently, the design of vector systems that allow the expansion of the payload of AAV vector systems even further might depend on the development of systems that rely on different mechanisms than trans-splicing and homologous recombination. Although purely speculative at this point, such systems might include trans-splicing of pre-mRNAs,^{71,†} or intein-mediated “protein trans-splicing.”⁷² Especially if such systems could be combined with presently available techniques, they might allow a significant expansion of genetic diseases that can be treated with AAV gene therapy.

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[†] Strictly speaking, trans-splicing and hybrid vector systems do not rely on trans-splicing of pre-mRNAs. Instead, in both systems a full-length mRNA is generated whose manufactured intron is then removed by regular *cis*-splicing to join the two exons.

REFERENCES

- Buning H. Gene therapy enters the pharma market: The short story of a long journey. *EMBO Mol Med* 2013;5:1–3.
- Hoggan MD, Blacklow NR, Rowe WP. Studies of small DNA viruses found in various adenovirus preparations: Physical, biological, and immunological characteristics. *Proc Natl Acad Sci U S A* 1966;55:1467–1474.
- Nault JC, Datta S, Imbeaud S, et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat Genet* 2015;47:1187–1193.
- Atchison RW, Casto BC, Hammon WM. Adenovirus-associated defective virus particles. *Science* 1965;149:754.
- Samulski RJ, Muzyczka N. AAV-mediated gene therapy for research and therapeutic purposes. *Ann Rev Virol* 2014;1:427–451.
- Berns KI, Adler S. Separation of two types of adeno-associated virus particles containing complementary polynucleotide chains. *J Virol* 1972;9:394–396.
- Hoggan MD. Adenovirus associated viruses. *Prog Med Virol* 1970;12:211–239.
- Rose JA, Berns KI, Hoggan MD, et al. Evidence for a single-stranded adenovirus-associated virus genome: Formation of a DNA density hybrid on release of viral DNA. *Proc Natl Acad Sci U S A* 1969;64:863–869.
- Kotin RM, Siniscalco M, Samulski RJ, et al. Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci U S A* 1990;87:2211–2215.
- Linden RM, Ward P, Giraud C, et al. Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci U S A* 1996;93:11288–11294.
- Samulski RJ, Zhu X, Xiao X, et al. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J* 1991;10:3941–3950.
- Tratschin JD, Miller IL, Carter BJ. Genetic analysis of adeno-associated virus: Properties of deletion mutants constructed *in vitro* and evidence for an adeno-associated virus replication function. *J Virol* 1984;51:611–619.
- Clark KR, Sferra TJ, Johnson PR. Recombinant adeno-associated viral vectors mediate long-term transgene expression in muscle. *Hum Gene Ther* 1997;8:659–669.
- Duan D, Sharma P, Yang J, et al. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol* 1998;72:8568–8577.
- Nakai H, Storm TA, Kay MA. Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in vivo*. *J Virol* 2000;74:9451–9463.
- Schnepp BC, Jensen RL, Chen CL, et al. Characterization of adeno-associated virus genomes isolated from human tissues. *J Virol* 2005;79:14793–14803.
- Nonnenmacher M, Weber T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther* 2012;19:649–658.
- de la Maza LM, Carter BJ. Molecular structure of adeno-associated virus variant DNA. *J Biol Chem* 1980;255:3194–3203.
- Srivastava A, Lusby EW, Berns KI. Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J Virol* 1983;45:555–564.
- Gerry HW, Kelly TJ Jr., Berns KI. Arrangement of nucleotide sequences in adeno-associated virus DNA. *J Mol Biol* 1973;79:207–225.
- Shao H, Lv Y, Ye J, et al. Isolation of a goose parvovirus from swan and its molecular characteristics. *Acta Virol* 2014;58:194–198.
- Wong FC, Spearman JG, Smolenski MA, et al. Equine parvovirus: Initial isolation and partial characterization. *Can J Comp Med* 1985;49:50–54.
- Diffoot N, Chen KC, Bates RC, et al. The complete nucleotide sequence of parvovirus Lull and localization of a unique sequence possibly responsible for its encapsidation pattern. *Virology* 1993;192:339–345.
- Siegl G. Physicochemical characteristics of the DNA of parvovirus Lu 3. *Arch Gesamte Virusforsch* 1973;43:334–344.
- Hermonat PL, Quirk JG, Bishop BM, et al. The packaging capacity of adeno-associated virus (AAV) and the potential for wild-type-plus AAV gene therapy vectors. *FEBS Lett* 1997;407:78–84.
- Dong JY, Fan PD, Frizzell RA. Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. *Hum Gene Ther* 1996;7:2101–2112.
- Brocchieri L, Karlin S. Protein length in eukaryotic and prokaryotic proteomes. *Nucleic Acids Res* 2005;33:3390–3400.
- Palfi A, Chadderton N, McKee AG, et al. Efficacy of codelivery of dual AAV2/5 vectors in the murine retina and hippocampus. *Hum Gene Ther* 2012;23:847–858.
- Allocca M, Doria M, Petrillo M, et al. Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invest* 2008;118:1955–1964.
- Lai Y, Yue Y, Duan D. Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome > or = 8.2 kb. *Mol Ther* 2010;18:75–79.
- Dong B, Nakai H, Xiao W. Characterization of genome integrity for oversized recombinant AAV vector. *Mol Ther* 2010;18:87–92.
- Wu ZJ, Yang HY, Colosi P. Effect of genome size on AAV vector packaging. *Mol Ther* 2010;18:80–86.
- Hirsch ML, Agbandje-McKenna M, Samulski RJ. Little vector, big gene transduction: Fragmented genome reassembly of adeno-associated virus. *Mol Ther* 2010;18:6–8.
- Hirsch ML, Li C, Bellon I, et al. Oversized AAV transduction is mediated via a DNA-PKcs-independent, Rad51C-dependent repair pathway. *Mol Ther* 2013;21:2205–2216.
- Ostedgaard LS, Rokhlina T, Karp PH, et al. A shortened adeno-associated virus expression cassette for CFTR gene transfer to cystic fibrosis airway epithelia. *Proc Natl Acad Sci U S A* 2005;102:2952–2957.
- McFarland TJ, Zhang Y, Atchaneeyaskul LO, et al. Evaluation of a novel short polyadenylation signal as an alternative to the SV40 polyadenylation signal. *Plasmid* 2006;56:62–67.
- Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor-VIII expressed in mammalian-cells. *J Biol Chem* 1988;263:6352–6362.
- Burton M, Nakai H, Colosi P, et al. Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. *Proc Natl Acad Sci U S A* 1999;96:12725–12730.
- Hu C, Lipshutz GS. AAV-based neonatal gene therapy for hemophilia A: Long-term correction and avoidance of immune responses in mice. *Gene Ther* 2012;19:1166–1176.
- Bostick B, Shin JH, Yue Y, et al. AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice. *Mol Ther* 2011;19:1826–1832.
- Gregorevic P, Blankinship MJ, Allen JM, et al. Systemic microdystrophin gene delivery improves skeletal muscle structure and function in old dystrophic mdx mice. *Mol Ther* 2008;16:657–664.
- Odom GL, Gregorevic P, Allen JM, et al. Gene therapy of mdx mice with large truncated dystrophins generated by recombination using rAAV6. *Mol Ther* 2011;19:36–45.
- Halbert CL, Allen JM, Miller AD. Efficient mouse airway transduction following recombination between AAV vectors carrying parts of a larger gene. *Nat Biotechnol* 2002;20:697–701.
- Sondergaard PC, Griffin DA, Pozsgai ER, et al. AAV-Dysferlin overlap vectors restore function in dysferlinopathy animal models. *Ann Clin Transl Neurol* 2015;2:256–270.
- Pryadkina M, Lostal W, Bourg N, et al. A comparison of AAV strategies distinguishes overlapping vectors for efficient systemic delivery of the 6.2 kb Dysferlin coding sequence. *Mol Ther Methods Clin Dev* 2015;2:15009.
- Trapani I, Colella P, Sommella A, et al. Effective delivery of large genes to the retina by dual AAV vectors. *EMBO Mol Med* 2014;6:194–211.
- Dyka FM, Boye SL, Chiodo VA, et al. Dual adeno-associated virus vectors result in efficient *in vitro*

- and *in vivo* expression of an oversized gene, MYO7A. *Hum Gene Ther Methods* 2014;25:166–177.
48. Lopes VS, Boye SE, Louie CM, et al. Retinal gene therapy with a large MYO7A cDNA using adeno-associated virus. *Gene Ther* 2013;20:824–833.
 49. Yan Z, Zak R, Zhang Y, et al. Inverted terminal repeat sequences are important for intermolecular recombination and circularization of adeno-associated virus genomes. *J Virol* 2005;79:364–379.
 50. Duan D, Yan Z, Yue Y, et al. Structural analysis of adeno-associated virus transduction circular intermediates. *Virology* 1999;261:8–14.
 51. Straus SE, Sebring ED, Rose JA. Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis. *Proc Natl Acad Sci U S A* 1976;73:742–746.
 52. Duan D, Yue Y, Yan Z, et al. A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation. *Nat Med* 2000;6:595–598.
 53. Yan Z, Zhang Y, Duan D, et al. Trans-splicing vectors expand the utility of adeno-associated virus for gene therapy. *Proc Natl Acad Sci U S A* 2000;97:6716–6721.
 54. Chao H, Sun L, Bruce A, et al. Expression of human factor VIII by splicing between dimerized AAV vectors. *Mol Ther* 2002;5:716–722.
 55. Duan D, Yue Y, Engelhardt JF. Expanding AAV packaging capacity with trans-splicing or overlapping vectors: A quantitative comparison. *Mol Ther* 2001;4:383–391.
 56. Reich SJ, Auricchio A, Hildinger M, et al. Efficient trans-splicing in the retina expands the utility of adeno-associated virus as a vector for gene therapy. *Hum Gene Ther* 2003;14:37–44.
 57. Sun L, Li J, Xiao X. Overcoming adeno-associated virus vector size limitation through viral DNA heterodimerization. *Nat Med* 2000;6:599–602.
 58. Choi VW, McCarty DM, Samulski RJ. Host cell DNA repair pathways in adeno-associated viral genome processing. *J Virol* 2006;80:10346–10356.
 59. Yan Z, Lei-Butters DC, Zhang Y, et al. Hybrid adeno-associated virus bearing nonhomologous inverted terminal repeats enhances dual-vector reconstruction of minigenes *in vivo*. *Hum Gene Ther* 2007;18:81–87.
 60. Trapani I, Toriello E, de Simone S, et al. Improved dual AAV vectors with reduced expression of truncated proteins are safe and effective in the retina of a mouse model of Stargardt disease. *Hum Mol Genet* 2015;24:6811–6825.
 61. Lai Y, Yue Y, Liu M, et al. Efficient *in vivo* gene expression by trans-splicing adeno-associated viral vectors. *Nat Biotechnol* 2005;23:1435–1439.
 62. Lai Y, Yue Y, Liu M, et al. Synthetic intron improves transduction efficiency of trans-splicing adeno-associated viral vectors. *Hum Gene Ther* 2006;17:1036–1042.
 63. Lostal W, Bartoli M, Bourg N, et al. Efficient recovery of dysferlin deficiency by dual adeno-associated vector-mediated gene transfer. *Hum Mol Genet* 2010;19:1897–1907.
 64. Ho M, Post CM, Donahue LR, et al. Disruption of muscle membrane and phenotype divergence in two novel mouse models of dysferlin deficiency. *Hum Mol Genet* 2004;13:1999–2010.
 65. Ghosh A, Yue Y, Lai Y, et al. A hybrid vector system expands adeno-associated viral vector packaging capacity in a transgene-independent manner. *Mol Ther* 2008;16:124–130.
 66. Ghosh A, Yue Y, Duan D. Viral serotype and the transgene sequence influence overlapping adeno-associated viral (AAV) vector-mediated gene transfer in skeletal muscle. *J Gene Med* 2006;8:298–305.
 67. Ghosh A, Yue Y, Duan D. Efficient transgene reconstitution with hybrid dual AAV vectors carrying the minimized bridging sequences. *Hum Gene Ther* 2011;22:77–83.
 68. Zhang Y, Duan D. Novel mini-dystrophin gene dual adeno-associated virus vectors restore neuronal nitric oxide synthase expression at the sarcolemma. *Hum Gene Ther* 2012;23:98–103.
 69. Lai Y, Thomas GD, Yue Y, et al. Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. *J Clin Invest* 2009;119:624–635.
 70. Koo T, Popplewell L, Athanasopoulos T, et al. Triple trans-splicing adeno-associated virus vectors capable of transferring the coding sequence for full-length dystrophin protein into dystrophic mice. *Hum Gene Ther* 2014;25:98–108.
 71. Yang Y, Walsh CE. Spliceosome-mediated RNA trans-splicing. *Mol Ther* 2005;12:1006–1012.
 72. Aranko AS, Volkmann G. Protein trans-splicing as a protein ligation tool to study protein structure and function. *Biomol Concepts* 2011;2:183–198.

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